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(54) Title: METHOD OF TREATING OR PREVENTING ABNORMAL CARDIAC CELL GROWTH BY INHIBITING THE 12–LIPOXYGENASE PATHWAY

(57) Abstract

The present invention is directed toward a method of treating or inhibiting abnormal cardiac growth. Specifically, the invention involves inhibition of the 12–lipoxygenase pathway or action of 12–lipoxygenase products, to reduce or eliminate 12–lipoxygenase pathway mediated cardiac hypertrophic effects at a cellular level.

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METHOD OF TREATING OR PREVENTING ABNORMAL CARDIAC CELL GROWTH BY INHIBITING THE 12-LIPOXYGENASE PATHWAY

This application claims priority from provisional application 60/086,147, filed May 20, 1998.

BACKGROUND OF THE INVENTION

1. Technical Field

This invention relates to a method for preventing or treating abnormal cardiac cell growth. The treatment or prevention is achieved by blockade of the 12-lipoxygenase pathway in the affected or potentially affected cells.

2. Description of the Background Art

Cardiac hypertrophy is an important indicator and often on early clinical sign of significant pathology in the heart. It is an adaptational state to prior hypertension and is a major risk factor associated with heart failure. The cardiac muscle has a large capacity for protein and nucleic acid synthesis since a high degree of turnover of these structural building blocks is necessary for maintenance of the cardiac muscle tissue. The normal process of continuous catabolism of heart proteins and their replacement allows the heart to more rapidly adjust to changes in the demands on the heart. However, when the balance of synthesis and degradation is disturbed, excessive compensation by the

heart in the form of surplus protein and nucleic acid synthesis can lead to cardiac enlargement and compromised contractile function of the heart. Cardiac enlargement may be genetically influenced or may be caused by overworking the heart secondary to disease, pharmacological agents or exercise, and eventually may lead to cardiac failure.

The first signs of cardiac hypertrophy usually are increases in protein and nucleic acid synthesis, as well as other changes in heart metabolism. Because the myocytes of the adult heart rarely undergo mitosis, enlargement of the heart generally is manifested by increases in cardiac muscle cell size rather than cell number. Some of the major symptoms of abnormal cardiac cell growth which can be detected easily in the laboratory include protein content increases, increase in cell size, and accumulation of fibrillar collagen in the extracellular space. Although cardiac muscle cells do not divide, often the connective tissue cells in the heart do increase in number in cardiac enlargement. This increase, and the accumulation of collagen and fibrillar fibronectin in the extracellular matrix lead to myocardial stiffness and ventricular dysfunction. Increase in fibrillar fibronectin is linked to the cell adhesion, migration and growth of cardiac muscle cells which is seen in the typical pattern of cardiac enlargement. It is the interstitial and perivascular fibrosis which accounts for abnormal cardiac stiffness and ultimately ventricular dysfunction.

Primary cultures of cardiomyocytes have been widely used and are recognized as a suitable in vitro model for cardiac hypertrophy at the cellular level.

Hefti et al., J. Mol. Cell Cardiol. 29:2873-2892 (1997). A great many enzymes, growth factors and cytokines have been postulated or shown to influence cardiac hypertrophy, however, the role of 12-lipoxygenase in this phenomenon previously has been undescribed.

Lipoxygenases are enzymes which produce active products, including 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) from arachidonic acid through stereospecific oxygenation. The normal physiological function of these enzymes is not well understood. However, 12-lipoxygenase (12-LO), the enzyme which catalyzes the oxygenation of arachidonic acid to 12(S)-HETE and (S)-12-hydroperoxyeicosatetraenoic acid (12(S)-HPETE), is known to exist in two forms (leukocyte-type and platelet-type) and to play a role in diseases such as atherosclerosis, diabetes and cancer. The mitogenic effects of 12(S)-HETE are similar to those of AII and are abrogated by pertussis toxin, implicating a G-protein mechanism.

12(S)-HETE has direct mitogenic effects in a Chinese hamster ovary (CHO) fibroblast cell line overexpressing the rat vascular type 1a angiotensin II (AT_{1a}) receptor. Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996). These 12(S)-HETE effects mimicked the angiotensin II (AII) mitogenic effects in these cells and led to a sustained increase in DNA synthesis as well as cell number. Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996). Furthermore, the addition of 12(S)-HETE to CHO-AT_{1a} cells led to a significant increase in the activity of the key growth-related kinases, mitogen

activated protein kinases (Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996)), and c-jun amino terminal kinase (Wen et al., Circ. Res. 81:651-655 (1997)). Therefore, angiotensin-II-mediated effects on mitogenesis are likely due to 12-LO products and 12(S)-HETE in particular. The LO pathway also plays a role in the chemotactic effects of platelet-derived growth factor. The products of the 12-LO pathway, are associated with the hypertrophic, hyperplastic, and mitogenic effects induced by AII. Wen et al., 271 Am. J. Physiol. (40 Cell Physiol.) C1212-C1220 (1996); (Natarajan et al., Hypertension 23:I142-I147 (1994)).

Although the precise mechanisms of 12(S)-HETE action are not clear, recent studies have shown that this LO product activates c-jun amino terminal kinase (JNK) (Wen et al., Circ. Res. 81:651-655 (1997)). JNK is a member of the MAP kinase family which is involved in cellular growth, inflammation, and apoptosis (Force et al., Circ. Res. 78:947-953 (1994)) and in cell cycle progression through G_1 (Olson et al., Science 269:1270-1272 (1995)). Evidence shows that JNK can serve as a positive or negative modulator of cell growth in different cells. Olson et al., 269 Science 1270-1272 (1995); Yan et al., 372 Nature 798-800 (1994). Stimulation of the 12-LO pathway in murine macrophages resulted in an increase of monocyte chemotaxis (Scheidegger et al., J. Biol. Chem., 272(34):21609-21615 (1997), presumably through modification of LDL. These activities link AII activation of 12-LO to atherosclerotic disease and cardiac hypertrophy.

Cardiac enlargement and the abnormal cell growth of cells in the heart is a serious health problem. Currently there is no adequate treatment for this condition. Consequently, a new method of treatment of abnormal cardiac cell growth would fill a need in the art.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a method of treating cardiac fibroblast cell growth and hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products. Another embodiment provides a method of preventing cardiac fibroblast cell growth and hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense oligonucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products. Yet another embodiment provides a method of reducing or

eliminating increased protein content in cardiac fibroblasts due to an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said fibroblasts with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense oligonucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the increased protein content resulting from said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a Western blot analysis of cardiac fibroblasts overexpressing 12-LO cDNA.

Figure 2 shows levels of 12(S)-HETE released into the medium bathing 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 3 provides data demonstrating ^{3}H-thymidine incorporation (DNA synthesis) and ^{3}H-leucine labeling (an indicator of protein synthesis) in 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 4 is a set of photomicrographs showing morphological changes in rat cardiac fibroblasts which have been transfected with 12-LO (Figure 4A) compared to mock-transfected rat cardiac fibroblasts (Figure 4B).

Figure 5 shows the forward scatter results of fluorescence-activated cell sorting comparisons of 12-LO-transfected and mock-transfected cells. In each case, 10^{6} cells were sorted.

Figure 6 shows the effect of 12-lipoxygenase overexpression in cardiac fibroblasts on MAP kinases and PAK activities. Arrows on the left side of the Figure indicate the substrates used for measurement of the respective kinase activities indicated at the right.

Figure 7 illustrates the effect of SB202190, a specific p38 MAP kinase inhibitor, on the protein content in 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 8 shows the effect of C_3 transferase pretreatment on ^3H-thymidine incorporation by 12-LO-transfected and mock-transfected cells.

Figure 9 shows Northern blot data indicating collagen $I\alpha_1$ mRNA levels in 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 10 provides data showing the level of soluble fibronectin released into the medium bathing 12-LO-transfected or mock-transfected cardiac fibroblasts and the increase of the fibrillar form of fibronectin resulting from 12-LO overexpression.

Figure 11 shows a significant reduction in cell growth induced by either AII or 12(S)-HETE (0.1μM) by DuP654, a specific 12(S)-HETE receptor blocker.

Figure 12 shows the inhibitory effect on leucine incorporation of baicalein, a specific 12-LO enzyme inhibitor, in cardiac fibroblasts.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Because 12(S)-HETE has several biological effects linked to cellular growth in vascular smooth muscle, CHO AT_{1a} cells and cardiac fibroblasts (Natarajan et

al., Hypertension 23:I142-I147 (1994); Wen et al., Am. J. Physiol. 211:C1212-C1220 (1996)), it is implicated in the etiology of several cardiovascular diseases, including cardiac hypertrophy. Applicants have discovered that 12-LO participates in a previously unknown growth promoting pathway in the heart by discovering that overexpression of 12-LO causes cardiac fibroblast cell growth. The present invention takes advantage of the 12-LO pathway effects which mediate cardiac hypertrophy to provide a new method of treating cardiac fibroblast cell growth and hypertrophy by blocking the expression, activity and/or products of 12-LO.

The harmful effects of 12-LO activation and 12(S)-HETE discussed above are ameliorated by blocking the production of 12(S)-HETE or its binding to specific receptors. For example, 12(S)-HETE receptor blockers include 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol, 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole, pertussis toxin, 12(S)-HETE analogs, antibodies to the 12(S)-HETE receptor and the like. Inhibitors of the 12-LO enzyme include panaxynol, phenidone, pioglitazone, substituted (carboxyalkyl)benzyl ethers, cinnamyl-3,4-dihydroxy-α-cyanocinnamate and the like. Compounds which serve as a structural analog for the enzyme may be useful. Some of these compounds have been described. Gorins et al. (J. Med. Chem. 39:4871-4878 (1996)).

The harmful effects of 12-LO and it products also may be ameliorated by reducing or eliminating 12-LO

expression using antisense or ribozyme methods. Ribozymes which cleave the 12-LO mRNA, preventing its expression, are useful in the invention, and may be constructed according to known methods. Antisense oligonucleotides which bind the 12-LO gene likewise are effective in preventing or reducing 12-LO expression and activity. These oligonucleotides may be constructed using methods known in the art.

To demonstrate the effects of 12-LO on cardiac fibroblasts, 12-LO was overexpressed using the calcium phosphate method in fibroblast cells from neonatal rat heart (kindly provided by Dr. Ping H. Wang, University of California, Irvine). Mouse leukocyte type 12-lipoxygenase cDNA was stably transfected into cardiac fibroblast cells (ML12-LO cells). Cardiac fibroblast cells were maintained in DME medium with 10% FBS containing 20 mM HEPES, pH 7.4, penicillin and streptomycin at 37°C in 5% CO_2 and 95% air. To generate the ML12-LO cells, cardiac fibroblasts were seeded at a density of 1 X 10^6 cells per 100 mm^2 dish. pcDNA1/ML12-LO vector and pPUR vector, a plasmid conferring resistance to puromycin, were cotransfected by the calcium phosphate DNA precipitation method according to manufacturer's instructions (Pharmacia Co.). The 12-LO overexpressing cell line is known as M4.7 cells. Cells transfected with the empty vector pcDNA 1 (Invitrogen) without the 12-LO cDNA insert (P3 cells) were used as a negative, mock-transfected control. Vectors were purified using an Endofree plasmid kit (Qiagen Co.). Forty-eight hours after transfection, the cells were split 1:15. Selection was then initiated with 2 μg/ml of puromycin to select

cells expressing resistance to this marker. Individual resistant clones were isolated 2-3 weeks later and expanded into cell lines. Transfected cells were maintained in medium containing 10% FBS, and 2 μg/ml puromycin.

Immunoblots were used to analyze the expression of the 12-LO protein. A polyclonal antibody against a peptide comprising amino acids 646-662 of porcine leukocyte 12-LO was raised in rabbits. The antibody showed excellent cross-reactivity with murine leukocyte 12-LO. For analysis, cells were lysed in PBS buffer (pH 7.4) containing 1% Triton X-100, 0.1% SDS and a standard protease inhibitor cocktail according to known methods. Lysates were sedimented and the supernatants were collected for assay. Protein concentration was determined by the Bradford method for this assay and all assays disclosed here. The protein (20 μg) was resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gel) and subsequently transferred to a polyvinylidine difluoride membrane. After the membrane was incubated in blocking buffer (Tropix Inc., Bedford) overnight, 12-LO antibody was added at a 1:1000 dilution. After incubation, an alkaline phosphatase coupled goat anti-rabbit secondary antibody was added at a 1:10,000 dilution. The protein bands were visualized using a chemiluminescence substrate and the Western Light Chemiluminescent detection system (Tropix Inc., Bedford). Figure 1 shows the Western blot analysis of these transfected cells. The results indicate several positive clones. It is clear that the M4.7 and M19 clones express much higher steady-state levels of the 12-LO protein compared to

mock-transfected clone P3 in which 12-LO protein was only slightly detectable. Clone M4.7 showed the greatest overexpression of 12-LO compared to the mock-transfected P3 cell line. (See Figure 1).

To evaluate cellular hypertrophy, these two cell lines (M4.7 and P3) were labeled with either ^{3}H-thymidine to evaluate DNA synthesis or with ^{3}H-leucine as an indicator of protein synthesis. For determination of ^{3}H-thymidine, ^{3}H-leucine or ^{3}H-uridine incorporation and protein content, cardiac fibroblast cells were grown in 6-well culture plates for 3 days. After twenty to twenty-four hours depletion with DME medium containing 20 mM HEPES, pH 7.4, 0.2% bovine serum albumin (BSA) and 0.4% fetal bovine serum (FBS), cells were continuously cultured in depletion medium containing 1 μCi/ml ^{3}H-thymidine, ^{3}H-leucine or ^{3}H-uridine. Approximately 24 hours later, the ^{3}H-isotope incorporation and protein content were measured. The medium was aspirated, cells were washed twice with 1 ml cold PBS solution and once with 1 ml 10% trichloroacetic acid (TCA). The cells were then incubated in fresh 1 ml 10% TCA at 4°C for 30 minutes. The TCA-insoluble material was washed twice with 95% ethanol. Fixed cellular material was solubilized in 0.1 N NaOH at 24°C for 2 hours. The sample was divided into 6 wells (3 wells for incorporated and protein content measurements; 3 wells for cell counting). The ^{3}H-isotope incorporation was determined by liquid scintillation spectrometry. Cells were counted with a Coulter Counter. The data was normalized as cpm/10^6 cells or μg protein/10^6 cells and expressed as fold increase over mock-transfected controls.

The data presented in Figure 2 show that 12-LO transfected cardiac fibroblasts release into the medium about 4 times the level of 12(S)-HETE than mock-transfected cells. 12(S)-HETE concentrations were measured by a specific radioimmunoassay with a sensitivity of 10 pg/ml and intraassay variation of 8%.

The results of ^{3}H-thymidine and ^{3}H-leucine labelling are shown in Figure 3. ^{3}H-Leucine incorporation is shown in the left panel. The overexpression of 12-LO resulted in increases in leucine incorporation about 2 fold (2.1 ± 0.1 fold; n=4; $p < 0.01$) over that in mock-transfected cells. The data in the right panel show that ^{3}H-thymidine incorporation increased more than 3 fold (3.4 ± 0.3 fold; n=4; $p < 0.01$) in cells overexpressing 12-LO compared to mock-transfected cells. Incorporation of ^{3}H-uridine in these 12-LO-transfected cells was over 5 fold greater than that seen in control cells (5.6 ± 0.7 fold; n=3; $p < 0.01$; data not shown). Protein content measurements indicated that cardiac fibroblasts overexpressing 12-LO contained 2.2 ± 0.3 fold more protein than controls when expressed as $\mu g/10^6$ cells (n=5; $p < 0.01$; data not shown). 12-LO expression did not lead to a true increase in cell number, however, these data clearly show that 12-LO products can result in dramatic increases in cell anabolism.

To further analyze hypertrophic effects of 12-LO overexpression, cell size was examined using hematoxylin eosin staining on cells in chamber slides. Cell size, nucleus size and nucleolus count were quantitated in mock- (Figure 4B) and 12-LO-transfected (Figure 4A) cells. Most of the mock-transfected cells

have one nucleolus. In contrast, 12-LO-transfected cells at the same magnification are bigger, the nuclei are larger and interestingly, they have a higher average number of nucleoli than control cells. See Figure 4. To quantiate this effect, twenty nuclei in Figure 4 were arbitrarily chosen and the long axis and numbers of nucleoli were measured. The data showed that the mean long axis of nuclei in 12-LO transfected cells was 6.4 ± 0.21 mm or nm compared to 4.15 ± 0.26 mm or nm in control cells. These cells were cultured in chamber slides and incubated at 37°C for 24 hours. After washing with PBS, the cells were fixed in 100% acetone and then stained with HE staining (hematoxylin for 5 minutes and eosin for 1 minute). The results indicate that the mean long axis of nuclei in 12-LO transfected cells was about 1.54 fold greater than that in control cells ($p < 0.001$). The analysis also demonstrated that the mean number of nucleoli was 2.95 in 12-LO transfected cells versus 1.5 in mock-transfected cells ($p < 0.001$). These results suggest that 12-LO overexpression leads to significant morphological changes consistent with cellular hypertrophy in rat cardiac fibroblasts.

The cell size increase in 12-LO transfected cells was confirmed using FACS analysis. The left panel of Figure 5 illustrates the forward scatter histogram of control and 12-LO transfected cells. The right panel of Figure 5 shows both 12-LO overexpressing and mock-transfected cells redrawn using a computer program. The average size of the 12-LO overexpressing cells was shifted to the right compared to that of control cells.

The effect of 12-LO overexpression on MAP kinases and PAK was evaluated since these signaling enzymes have been linked to cell growth, cell apoptosis and focal adhesion activity. ERK1/2, JNK-1 and p38 activity was measured with an immune complex kinase assay according to methods known in the art. Growth of the cells was arrested by incubation in depletion DME medium containing 0.2% BSA, 0.4% FCS, 20 mM HEPES, pH 7.4, for 24 hours. After washing twice with cold PBS, the cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 50 mM NaF, 10 mM Na pyrophosphate, 2.5% glycerol, 1% NP-40 and 1 mM Na_3VO_4 and the protease inhibitors PMSF, leupeptin and aprotinin. The lysate was sedimented at 14,000 xg at 4°C for 10 minutes. The lysate protein (50 μg) was immunoprecipitated with JNK, p38 or ERK antibodies in lysis buffer and the mixture incubated under rotation at 4°C overnight, followed by addition of the solution to 60 μl protein A Sepharose beads. After 1 hour incubation with protein A Sepharose at 4°C, the beads were washed 4 times with buffer and pelleted. The pelleted beads were resuspended in 60 μl kinase buffer containing substrates as follows: 2 μg GST-c-Jun(aa 1-79) for the JNK assay, 2 μg ATF-2 for the p38 assay or 2 μg MBP for the ERK assay and 20 μM ATP containing 5 μCi [γ-^{32}P]ATP. After 30 minutes at 30°C, the reaction was stopped and samples were resolved on 12% SDS-polyacrylamide gels followed by autoradiography.

The protein kinase activity studies showed that overexpression of 12-LO enhanced extracellular signal-regulated kinase (ERK) activity, cJun NH_3-terminal kinase (JNK) activity, p38 MAP kinase (p38)

activity and p21-activated kinase (PAK) activity. See Figure 6. These data clearly demonstrate that overexpression of 12-LO causes cardiac fibroblast cell growth, showing that 12-LO participates in a previously unknown growth promoting pathway to the heart. The data in Figure 6 clearly show that the overexpression of 12-LO results in activation of JNK, ERK, p38 and PAK. Overexpression of 12-LO stimulated ERK 4.1 ± 0.5 fold ($n=3$; $p < 0.01$), p38 2.2 ± 0.3 fold ($n=3$; $p < 0.02$) and JNK 2.9 ± 0.5 fold ($n=3$, $p < 0.02$), respectively.

Figure 7 presents data showing the effect of SB202190, a specific p38 MAP kinase inhibitor, on the protein content of cardiac fibroblast cells. Comparing the data for the 12-LO transfected cells and mock-transfected controls shows that the overexpression of 12-LO results in increases in protein content of about 2 fold. The p38 inhibitor had no significant effect on protein content in mock-transfected cells, implying that p38 MAP kinase activation may be important for 12-LO-induced protein content increases, but completely blocked the protein content increase induced by 12-LO overexpression. In contrast, the compound PD58059, an inhibitor of MEK, had no effect on leucine incorporation either in 12-LO-transfected or mock-transfected cells (data not shown). This clearly demonstrates the effect that abnormally increased 12-LO expression has on cardiac fibroblasts, and the relationship of 12-LO to p38 MAP kinase, a known enzyme in the signal pathway which causes cardiac enlargement.

Previous evidence has shown that arachidonic acid and its metabolites can activate small molecular weight

GTP-binding proteins. To elucidate the effect of the small molecular weight GTP-binding protein, Rho, on ^{3}H-thymidine incorporation, M4.7 and P3 cardiac fibroblasts were pretreated with the specific Rho GTPase inhibitor, ADP-ribosyltransferase C_3 (C_3 transferase; 10 μg/ml) for 24 hours. C_3 transferase treatment reduced DNA synthesis by 34% in the 12-LO overexpressing cardiac fibroblast cells but not in mock transfected cardiac fibroblast cells (see Figure 8). These results suggest that 12-LO-induced increases in cardiac fibroblast growth is mediated at least in part by activation of small molecular weight GTP-binding proteins such as Rho.

A collagen Iα_1 cDNA probe was used to detect collagen mRNA content in these cells as an indicator of another symptom in cardiac hypertrophy. Figure 9 illustrates the results. As shown in the top panel, the Northern Blot detected two collagen bands at 4.6 kb and 5.6 kb. This is consistent with the published data for this probe. The lower two panels show the densities of these two bands indicating that the overexpression of 12-LO increased mRNA content of these two bands about 4 fold over that in mock-transfected cells.

To evaluate the effect of 12-LO expression on matrix production, soluble fibronectin released into the medium of cardiac fibroblasts transfected or mock-transfected with 12-LO was measured with a specific ELISA assay as yet another test of the relationship between 12-LO and cardiac hypertrophy. Because the presence of fibrillar fibronectin is more closely related to cardiac hypertrophy, measurements

were taken not only for fibronectin released into medium (soluble fibronectin), but also 1% deoxycholate insoluble fibronectin (fibrillar fibronectin).

Cells at 80% to 90% confluence were depleted with medium containing 0.2% BSA and 0.4% FCS for 24 hours. The supernatants were assayed for released fibronectin. Washed cell layers were extracted with 1% deoxycholate. Deoxycholate extractions were performed in 0.02 M Tris buffer, pH 8.3, containing 2 mM phenylmethylsulfonilfluoride (PMSF), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylmaleimide and 2 mM iodoacetic acid. For sequential extraction, cell layers were scraped into 1% deoxycholate and sedimented at 17,000 rpm for 20 minutes. Deoxycholate-insoluble material was solubilized at 70°C in 4% SDS. The fibronectin in supernatants was classified as soluble fibronectin, while the 1% deoxycholate-insoluble material was classified as fibrillar. Fibronectin in all samples was determined by a double-antibody sandwich enzyme-linked immunosorbent assay using the methods provided by the manufacturer (DACO Corp., Carpinteria, CA). A polyclonal rabbit anti-human fibronectin (1:1000) was used as the coating antibody, and the detection antibody was a peroxidase-conjugated rabbit anti-human fibronectin (1:2000).

The results show that overexpression of 12-LO increases soluble fibronectin release into the medium about 3.7 fold over that in mock-transfected cells ($p < 0.01$) and increases fibrillar fibronectin 3.4 times compared to mock-transfected cells ($p < 0.01$). See Figure 10. This suggests that more surface-bound

fibronectin assembled into large fibrils in cells overexpressing 12-LO.

The results of these experiments on cell protein content, cell size, collagen production and fibrillar fibronectin clearly indicate that overexpression of 12-LO causes both cardiac fibroblast cell hypertrophy and matrix production, and demonstrate that 12-LO participates in a heretofore unrecognized growth-promoting pathway in the heart. The present invention therefore provides a method of treating or preventing abnormal cardiac cell growth by inhibiting the effects of 12-LO and its products. This may be accomplished by antagonizing or blocking the receptors for the 12-LO product, 12(S)-HETE, with a 12(S)-HETE receptor blocker such as 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 12(S)-HETE receptors, antibodies to the 12(S)-HETE receptor and the like. Reducing or eliminating the production of 12(S)-HETE through a 12-LO enzyme inhibitor, for example, panaxynol, a polyacetylene compound isolated from ginseng (Powell et al., Science 245:186-188 (1989)), phenidone, 5,6,7-trihydroxyflavone (baicalein), pioglitazone, substituted (carboxyalkyl)benzyl ethers such as those disclosed in Gorins et al., J. Med. Chem. 39:4871-4878 (1996), cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) and the like also forms part of the invention.

Expression of 12-LO enzyme may be prevented using antisense or ribozyme methods according to the invention. Antisense oligonucleotides which bind 12-LO genetic material are useful to prevent or reduce 12-LO activity in cardiac fibroblasts. This invention therefore provides a method of controlling cardiac cell growth and hypertrophy employing antisense. Oligonucleotides may be constructed using any suitable method known in the art. Ribozymes designed to specifically cleave 12-LO mRNA are provided by the present invention. Methods known in the art may be used to construct ribozymes which bind and cleave 12-LO mRNA. He cardiac cell growth and hypertrophic effects of 12-LO are thereby reduced or eliminated.

The determination of appropriate, well-tolerated dosage forms for administration to humans for use in the present invention is within the ordinary skill in the art. Such dosage forms include tablets, capsules, syrups, suspensions, drops, injectable solutions, lozenges, implants, transdermal patches, and other dosage forms well known in the art for enteral or parenteral administration. Based on in vitro experiments on the effect of 12(S)-HETE receptor blocking drugs on 12(S)-HETE binding, a dose of between about 0.5 and about 30 mg/kg/day would be effective in blocking 12(S)-HETE receptors in humans in vivo, and preferably from about 1 to about 10 mg/kg/day. Similar dosages may be given when 12-LO inhibitors are used. Methods of administering ribozymes or antisense oligonucleotides may be designed according to any suitable known method.

EXAMPLES

Example 1

Reduction of cell growth induced by AII or 12(S)-HETE.

DuP654, a selective 12(S)-HETE receptor antagonist, significantly reduced cell growth induced by either AII or 12(S)-HETE at a concentration of 0.1 mM in CHO-AT_{1a} cells in vitro. Complete inhibition of 12(S)-HETE induced mitogenic effects was seen. See Figure 11.

Example 2

Inhibition of serum-induced leucine incorporation by cardiac fibroblasts.

Baicalein, a specific inhibitor of 12-lipoxygenase, inhibited leucine incorporation in cardiac fibroblasts. The inhibition was both highly significant and dose-dependant ($P<0.005$ at 1μM; $P<0.001$ at 10 μM). See Figure 12. These data clearly demonstrate that blockade of the 12-LO pathway meaningfully reduces the indicia of cell growth in mammalian cardiac fibroblasts.

CLAIMS

1. A method of treating cardiac fibroblast cell growth and hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

2. The method according to claim 1, wherein the compound is a 12-lipoxygenase inhibitor.

3. The method according to claim 2, wherein the 12-lipoxygenase inhibitor is selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl-3,4-dihydroxy-α-cyanocinnamate.

4. The method according to claim 1, wherein the compound is a 12(S)-HETE receptor blocker.

5. The method according to claim 4, wherein the 12(S)-HETE receptor blocker is selected from the group consisting of 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having

affinity for the binding site on the 12(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

6. The method according to claim 1, wherein the compound is a 12-lipoxygenase antisense nucleotide.

7. The method according to claim 1, wherein the compound is a 12-lipoxygenase ribozyme.

8. A method of controlling cardiac fibroblast cell growth and preventing hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme, and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

9. The method according to claim 8, wherein the compound is a 12-lipoxygenase inhibitor.

10. The method according to claim 9, wherein the 12-lipoxygenase inhibitor is selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl-3,4-dihydroxy-α-cyanocinnamate.

11. The method according to claim 8, wherein the compound is a 12(S)-HETE receptor blocker.

12. The method according to claim 11, wherein the 12(S)-HETE receptor blocker is selected from the group consisting of 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 12(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

13. The method according to claim 8, wherein the compound is a 12-lipoxygenase antisense nucleotide.

14. The method according to claim 8, wherein the compound is a 12-lipoxygenase ribozyme.

15. A method of reducing or eliminating increased protein content in cardiac fibroblasts due to an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said fibroblasts with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the increased protein content resulting from said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

16. The method according to claim 15, wherein the compound is a 12-lipoxygenase inhibitor.

17. The method according to claim 16, wherein the 12-lipoxygenase inhibitor is selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl-3,4-dihydroxy-α-cyanocinnamate.

18. The method according to claim 15, wherein the compound is a 12(S)-HETE receptor blocker.

19. The method according to claim 18, wherein the 12(S)-HETE receptor blocker is selected from the group consisting of 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 12(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

Western Blot of 12-LO Protein Levels in Cardiac Fibroblasts Overexpressing Mouse 12-LO cDNA

<= 12-LO 72kD

Clones M19 M4.7 P3

Fig. 1

Fig. 2

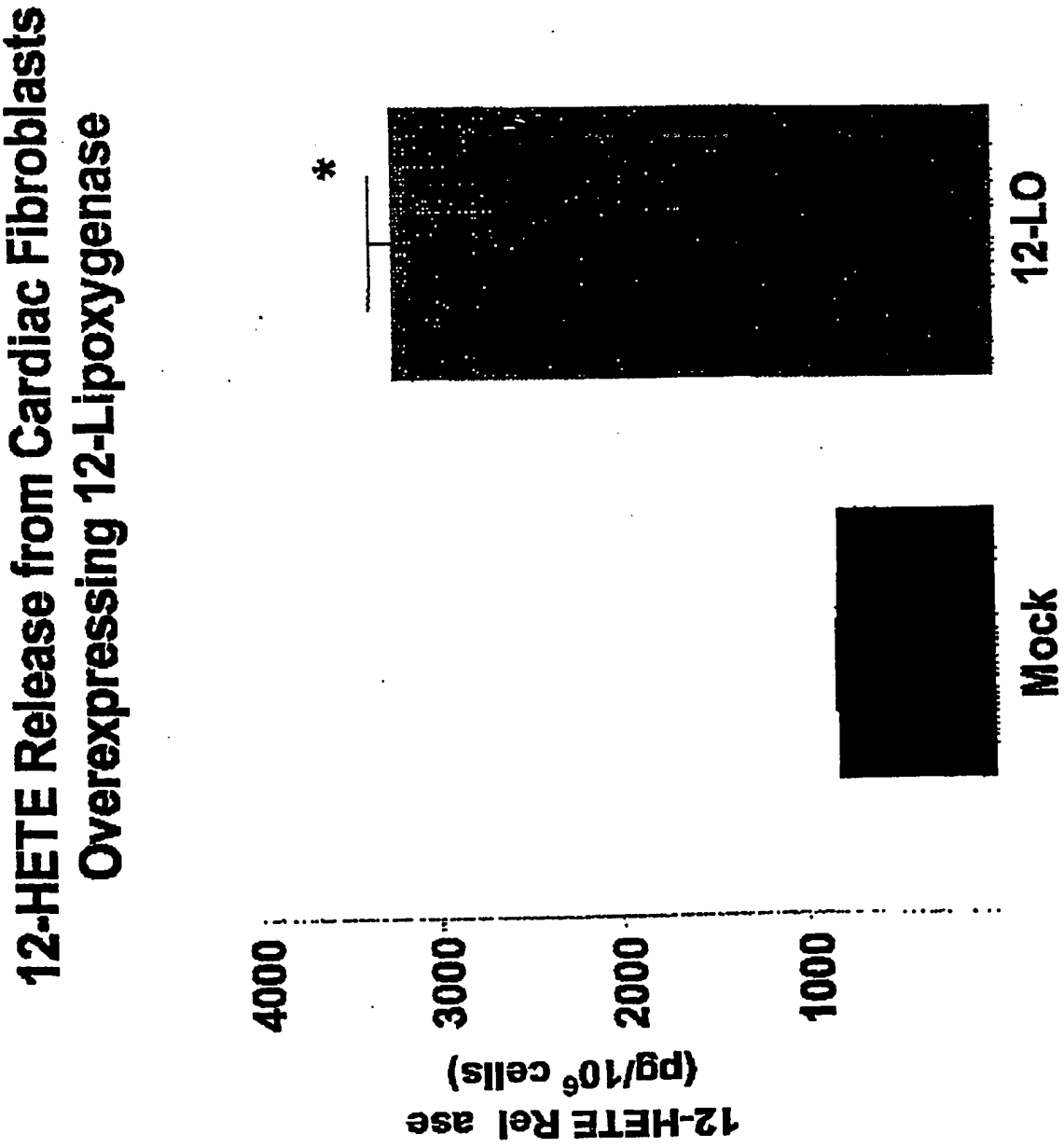
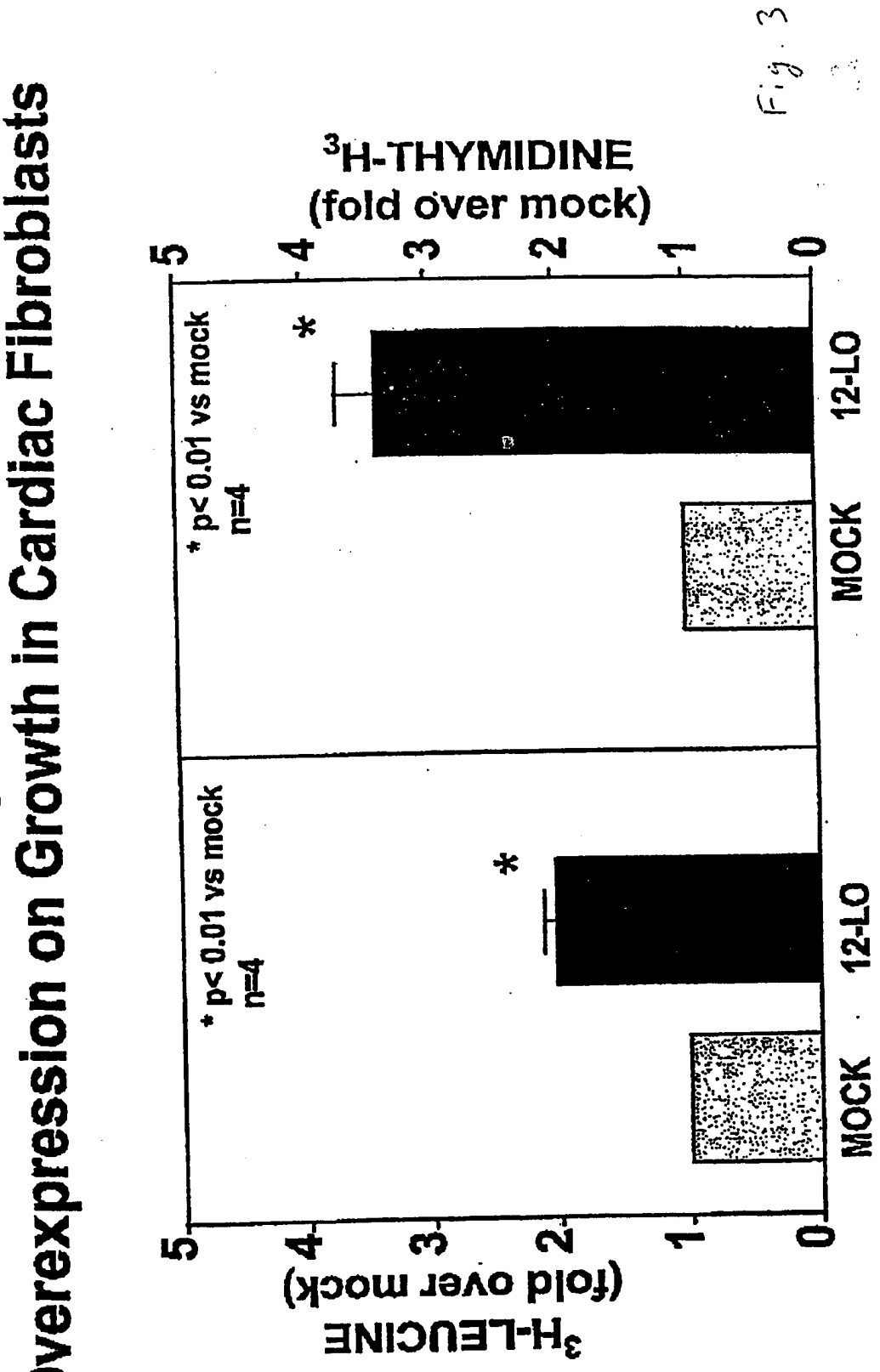


Fig. 3



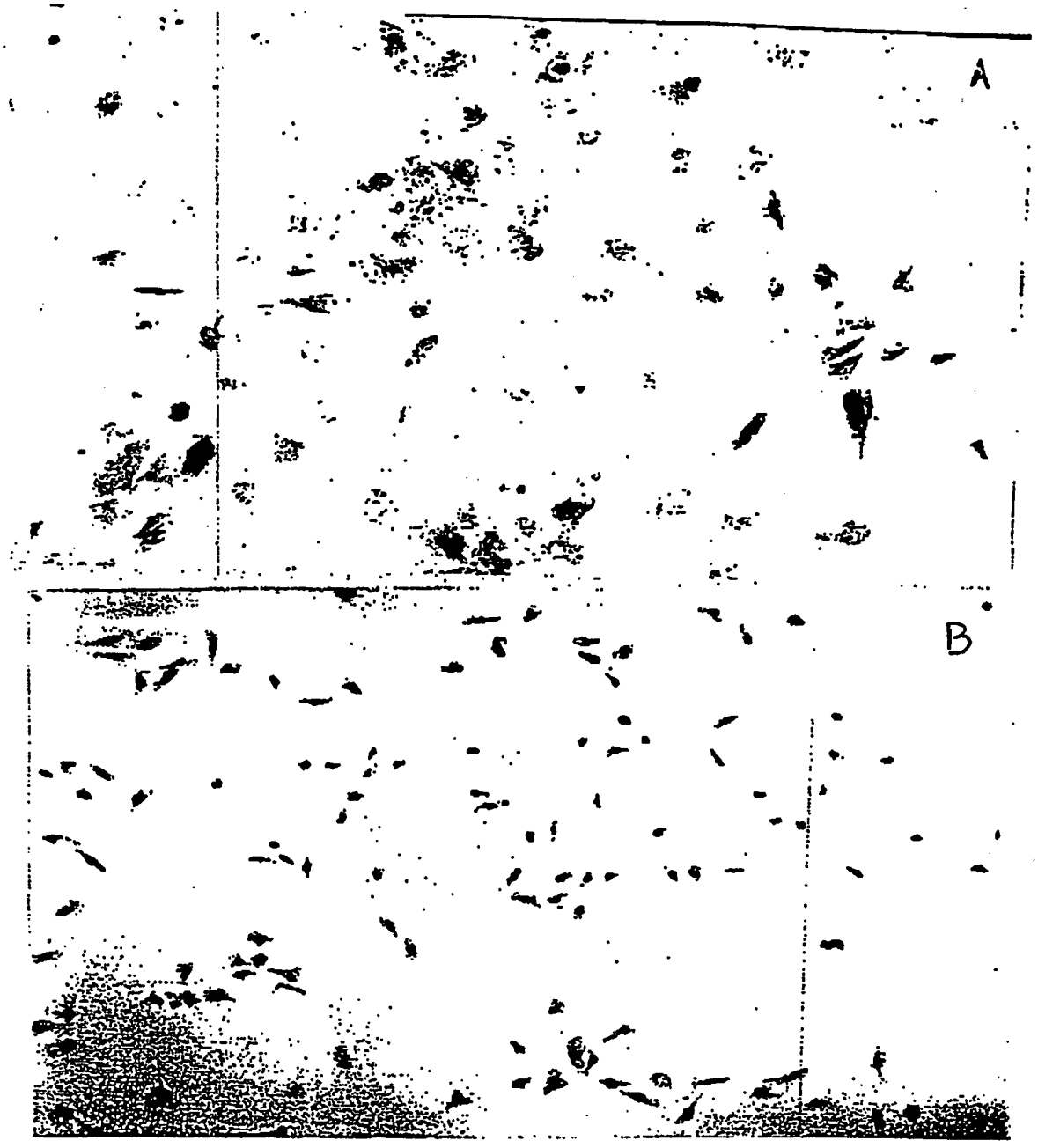


Fig. 4

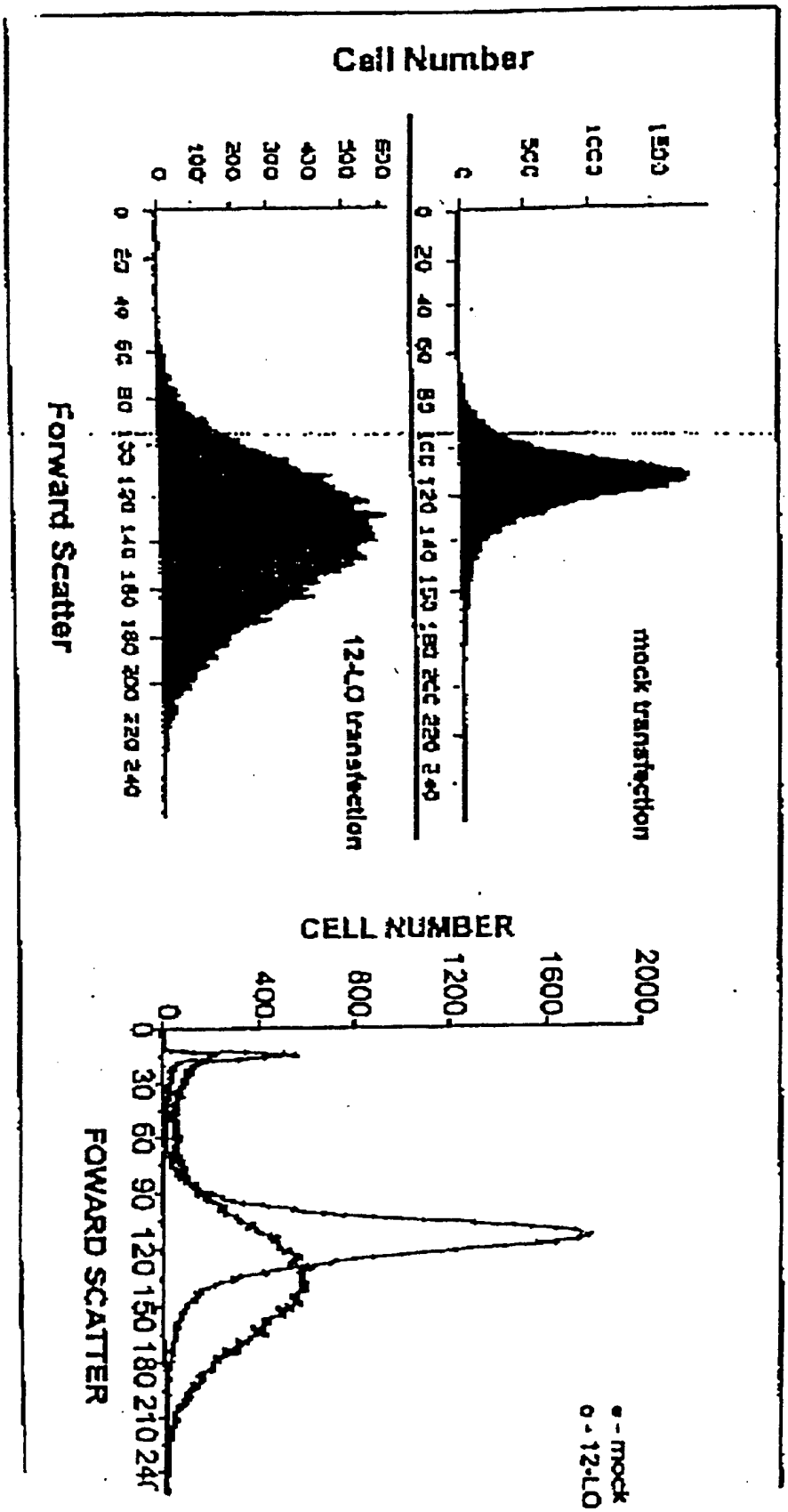

Cell Number
mock transfection
12-LO transfection
Forward Scatter
CELL NUMBER
FOWARD SCATTER
• - mock
o - 12-LO

Fig 5

Overexpression of 12-LO Stimulates the MAP Kinases and PAK Activities in Cardiac Fibroblast Cells.

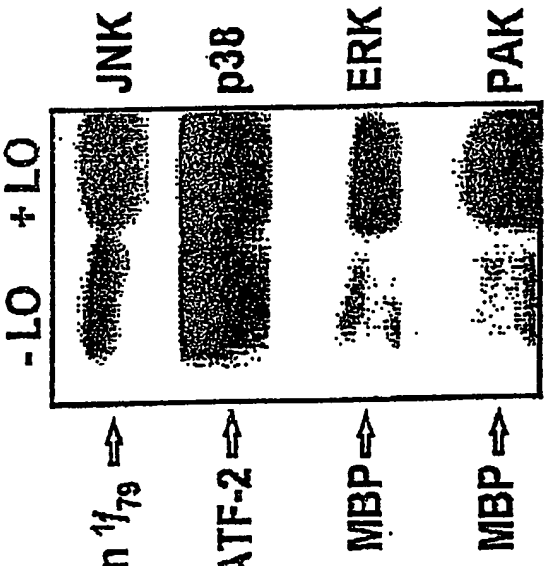


Fig. 6

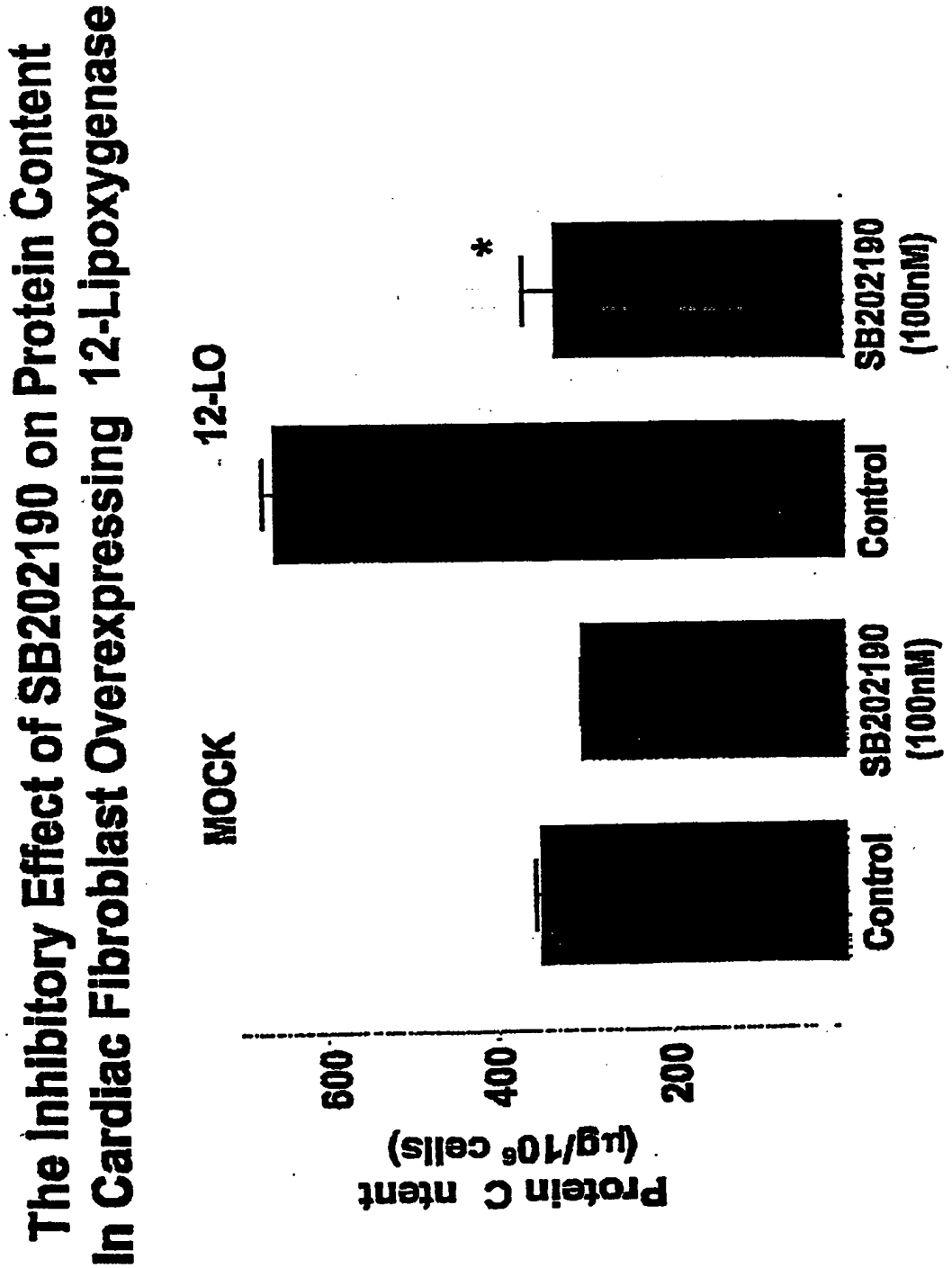

The Inhibitory Effect of SB202190 on Protein Content
In Cardiac Fibroblast Overexpressing 12-Lipoxygenase
Protein Content
(µg/10⁶ cells)
600
400
200
MOCK
12-LO
Control
SB202190 (100nM)
Control
SB202190 (100nM)
*

Fig. 7

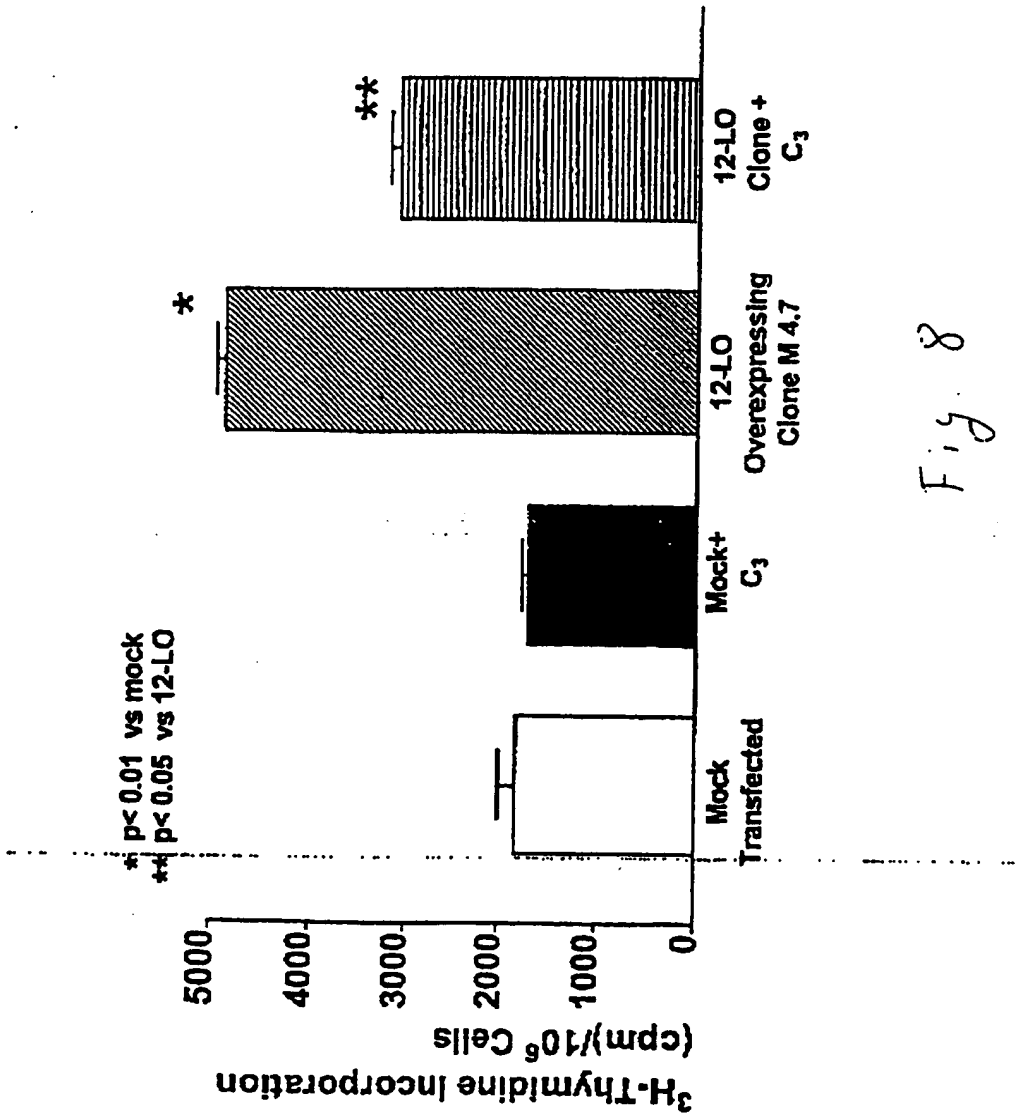
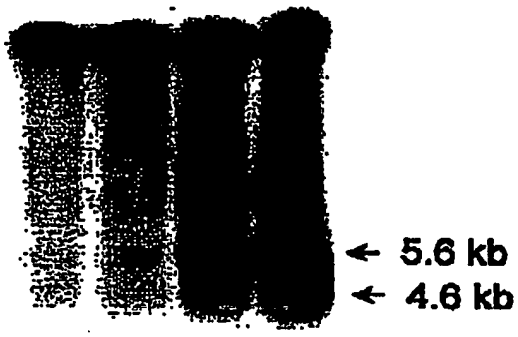


Fig 8

Overexpression of 12-Lipoxygenase Increases Collagen Type Iα_1 mRNA Level in Cardiac Fibroblast Cell



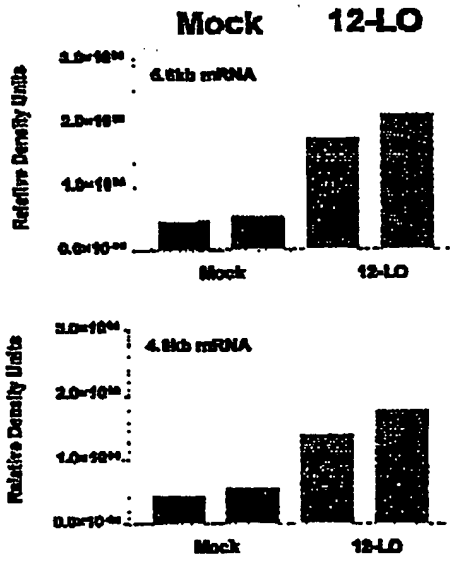


Fig. 9

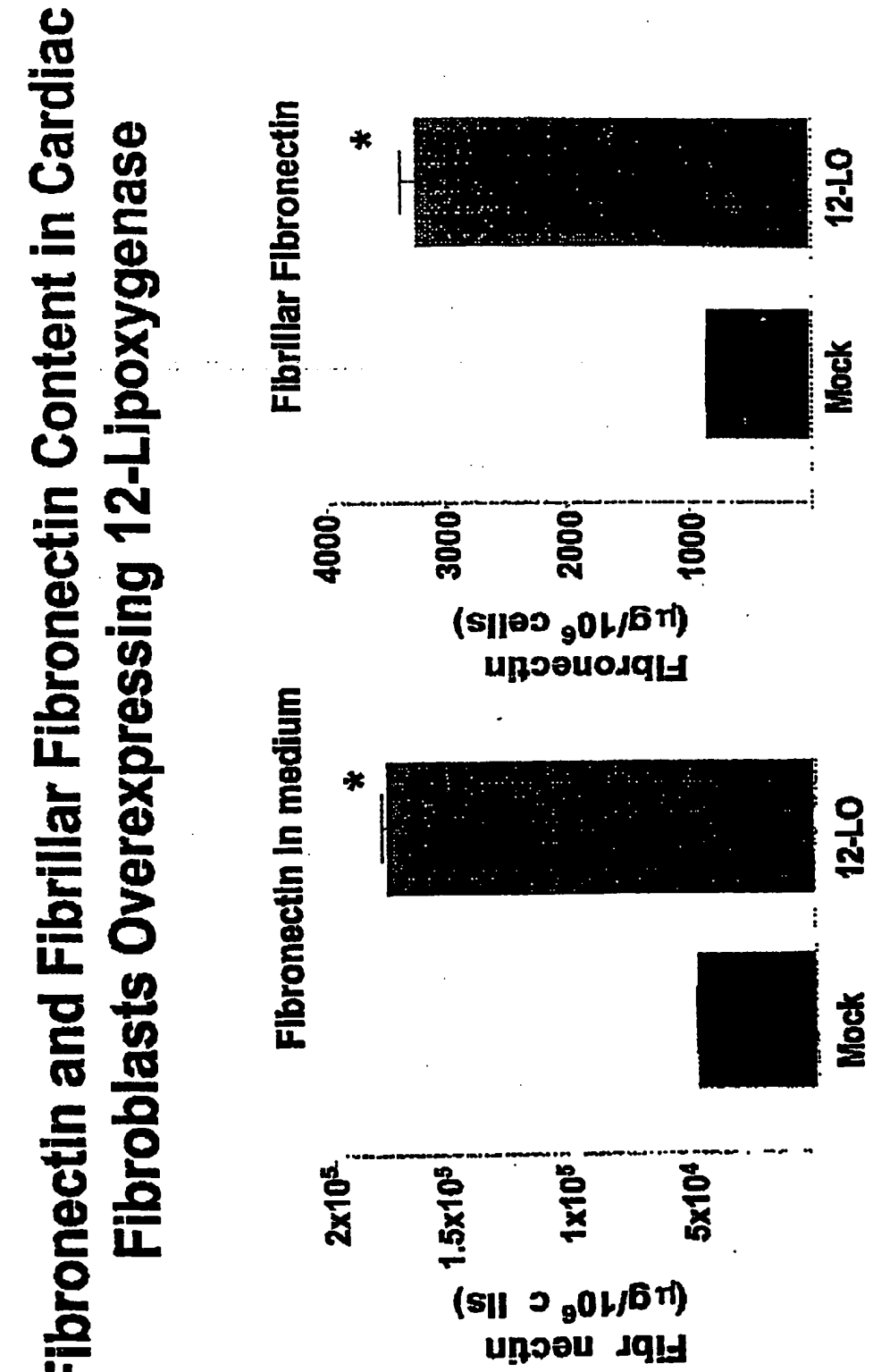


Fig 10

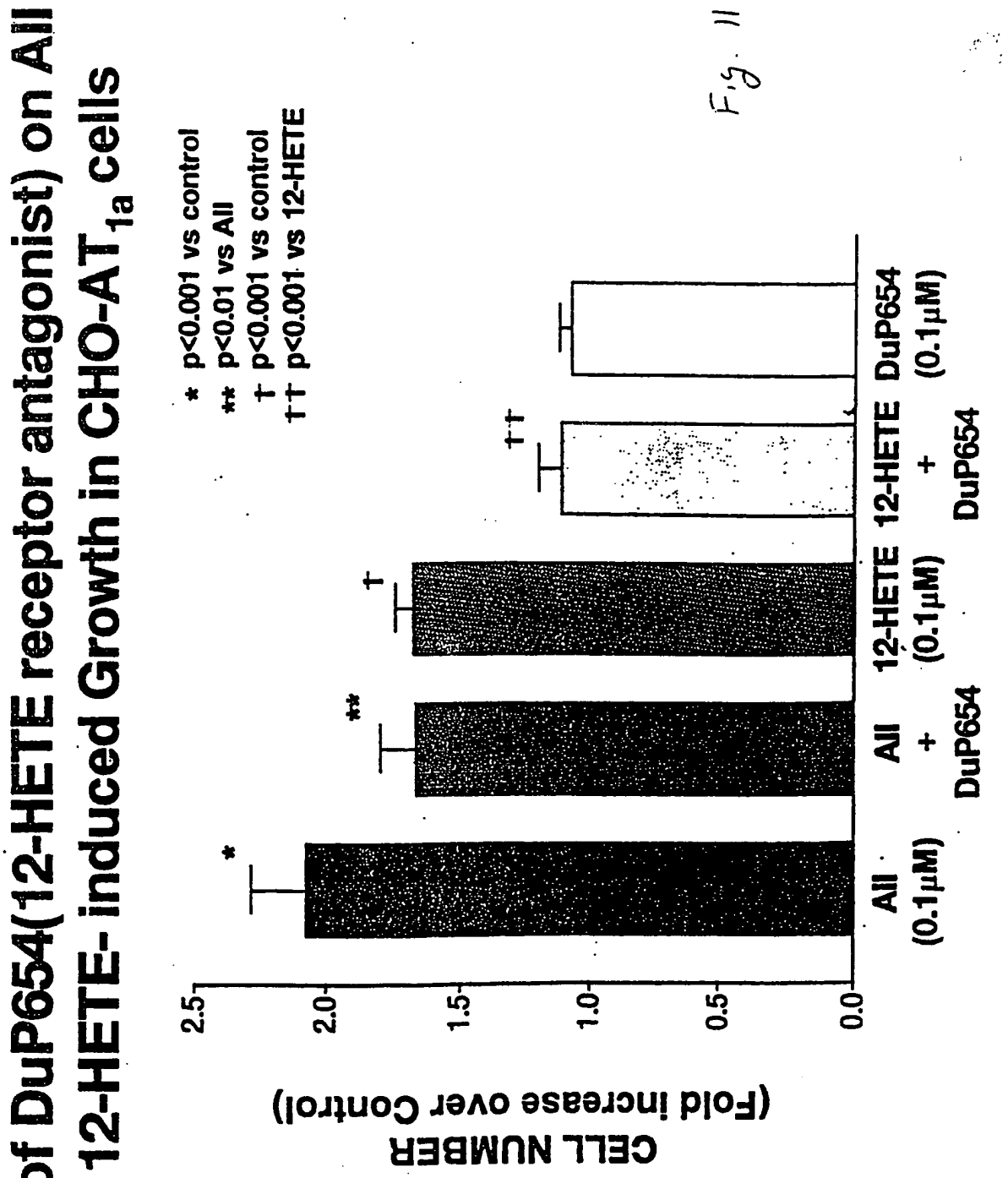


Fig. 11

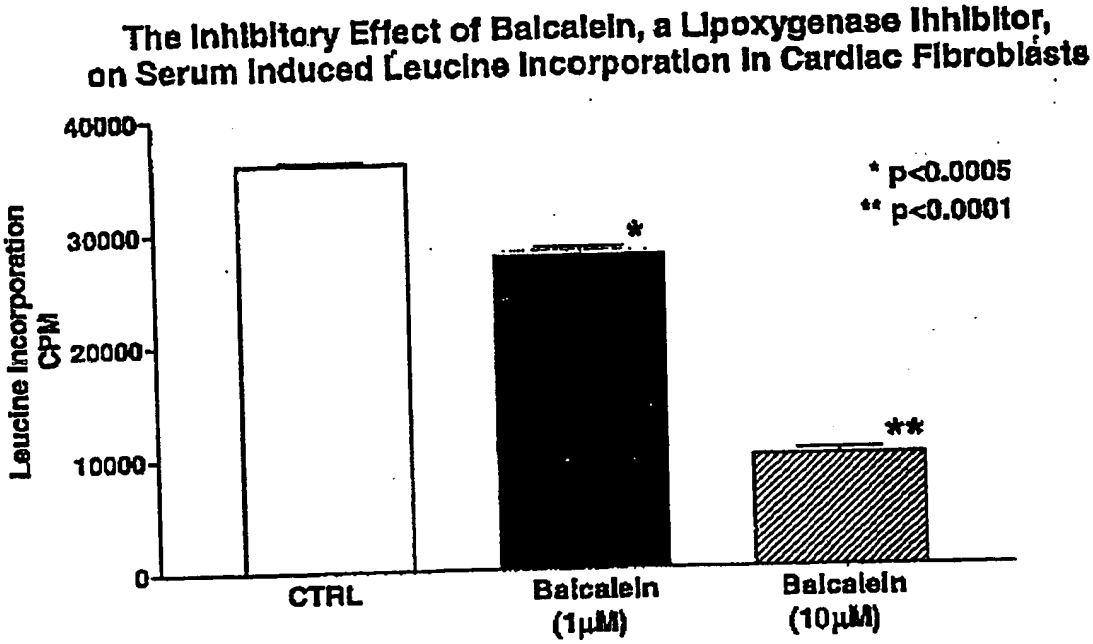


Fig. 12

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(54) Title: METHOD OF TREATING OR PREVENTING ABNORMAL CARDIAC CELL GROWTH BY INHIBITING THE 12-LIPOXYGENASE PATHWAY

(57) Abstract

The present invention is directed toward a method of treating or inhibiting abnormal cardiac growth. Specifically, the invention involves inhibition of the 12-lipoxygenase pathway or action of the 12-lipoxygenase products, to reduce or eliminate 12-lipoxygenase pathway mediated cardiac hypertrophic effects at a cellular level. Useful compounds are selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl1-3,4-dihydroxy-a-cyanocinnamate, 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol(DuP654), 2-N-butyl-4-cholo-5-hydroxymethyl1-1-[2'-(1H-tetrazol-5-zl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 23(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/11115

A. CLASSIFICATI N OF SUBJECT MATTER
IPC 6 A61K31/44 A61K31/415 A61K31/35 A61K31/275 A61K31/20
A61K31/05 A61K31/04

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BROTEN ET AL.: "Losartan and enalapril prevent cardiac hypertrophy and intramural coronary arterial hypertrophy and fibrosis in a low renin model of hypertension." FASEB JOURNAL, vol. 8, no. 4-5, 1994, page A310 XP002117416 abstract --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
3 November 1999	- 8. 02. 00

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 99/11115

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DOMINICZAK A. F.; DEVLIN A. M.; LEE W. K.; ANDERSON N. H.; BOHR D. F.; REID J. L.: "Vascular smooth muscle polyploidy and cardiac hypertrophy in genetic hypertension." HYPERTENSION, vol. 27, no. 3 pt 2, 1996, pages 752-759, XP002117417 abstract; figures 1,3,4 page 756, column 2, paragraph 1 ---	1
X	GUO, ZHAO-GUI; LING, QI; SU, ZHI: "Mechanism of losartan in the regression of cardiac hypertrophy." FASEB JOURNAL, vol. 12, no. 5, 1998, page a709 XP002117418 abstract ---	1
X	NATARAJAN, RAMA; GONZALES, NOE; LANTING, LINDA; NADLER, JERRY: "Role of the lipoxygenase pathway in angiotensin II-induced vascular smooth muscle cell hypertrophy." HYPERTENSION, vol. 23, no. 1, 1994, pages I142-I147, XP002117419 abstract; figures 2-4 page I145, column 1, paragraph 3 -page I146, column 1, paragraph 2 ---	1,4,5,8, 11,15, 18,19
X	DETHLEFSEN, SANDRA M. ; SHEPRO, DAVID; D'AMORE, PATRICIA A.: "Arachidonic acid metabolites in bFGF-, PDGF-, and serum-stimulated vascular cell growth." EXPERIMENTAL CELL RESEARCH, vol. 212, no. 2, 1994, pages 262-273, XP002117420 abstract page 262, column 2, paragraph 3 -page 263, column 1, paragraph 1; figures 1-12 see results ---	1,4,5,8, 11,15, 18,19
X	WO 96 40256 A (SEARLE & CO ;MCMAHON ELLEN G (US); OLINS GILLIAN M (US); SCHUH JOS) 19 December 1996 (1996-12-19) page 8, line 3 -page 11, line 27 page 130, compound 325 --- -/--	1

5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Structure-activity relati nship for potentiation of EGF-dependent mitogenesis by oxygenated metabolites of linoleic acid" ARCH. BIOCHEM. BIOPHYS. (1994), 311(2), 286-92, XP002117421 abstract page 286, column 1, paragraph 1 -page 287, column 1, paragraph 2 page 288, column 1, paragraph 1 -page 289, column 2, paragraph 1; figure 2; table 1 ---	1,4,5,8, 11,15, 18,19
A	BAILEY ET AL.: "15-lipoxygenase induction as an index of oxidative stress and atherogenesis" BIOCHEM. SOC. TRANS., vol. 21, no. 4, 1993, page 406S XP002117422 the whole document ---	1,4,5,8, 11,15, 18,19
X	EP 0 339 671 A (SUNTORY LTD) 2 November 1989 (1989-11-02) page 2, line 1-50 ---	1,4
X,P	YESHAO, WEN ; GU, JIALI ; WANG, PING H.; NADLER, JERRY L.: "Overexpression of 12 lipoxygenase causes cardiac fibroblast cell growth." HYPERTENSION, vol. 32, no. 3, 1998, page 630 XP002117423 abstract ---	1
P,X	WO 99 18956 A (HOPE CITY) 22 April 1999 (1999-04-22) page 19, line 7-17; example 2 ---	1,2,4
X	US 5 102 912 A (STREBER AUGUST S) 7 April 1992 (1992-04-07) column 2, line 12 -column 4, line 5; claims 1,6,8 -----	1,4,5,8, 11,12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 11115

Box I Observations where certain claims were found unsearchabl (Continuati n of it m 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-19 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,4,5,8,11,15,18,19 (all partially)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of 13-(S)-hydroxyoctadecadienoic acid and other 12-(S)-HETE analogs for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

2. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of 2-phenylmethyl-1-naphthol for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

3. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of losartan for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

4. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of pertussis toxin for treating and controlling cardiac fibroblast cell growth and hypertrophy in a cell.

5. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of peptides and peptide analogs having affinity for the binding site one the 12 (S)-HETE receptor and antibodies to the 12(S)-HETE receptor for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

6. Claims: 1 (partially), 6, 8 (part.), 13, 15 (part.)

   Use of a 12-lipoxygenase antisense nucleotides for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

FURTHER INFORMATION CONTINUED FR M PCT/ISA/ 210

7. Claims: 1 (partially), 7, 8 (part.), 14

Use of a 12-lipoxygenase ribozyme for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

8. Claims: 1-3,8-10,15-17 (all partially)

Use of pioglitazone for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

9. Claims: 1-3,8-10,15-17 (all partially)

Use of panaxynol for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

10. Claims: 1-3,8-10,15-17 (all partially)

Use of phenidone for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

11. Claims: 1-3,8-10,15-17 (all partially)

Use of baicalein for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

12. Claims: 1-3,8-10,15-17 (all partially)

Use of cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

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(57) Abstract

The present invention is directed toward a method of treating or inhibiting abnormal cardiac growth. Specifically, the invention involves inhibition of the 12-lipoxygenase pathway or action of the 12-lipoxygenase products, to reduce or eliminate 12-lipoxygenase pathway mediated cardiac hypertrophic effects at a cellular level. Useful compounds are selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl1-3,4-dihydroxy-a-cyanocinnamate, 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol(DuP654), 2-N-butyl-4-cholo-5-hydroxymethyl1-1-[2'-(1H-tetrazol-5-zl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 23(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

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METHOD OF TREATING OR PREVENTING ABNORMAL CARDIAC CELL GROWTH BY INHIBITING THE 12-LIPOXYGENASE PATHWAY

This application claims priority from provisional application 60/086,147, filed May 20, 1998.

BACKGROUND OF THE INVENTION

1. Technical Field

This invention relates to a method for preventing or treating abnormal cardiac cell growth. The treatment or prevention is achieved by blockade of the 12-lipoxygenase pathway in the affected or potentially affected cells.

2. Description of the Background Art

Cardiac hypertrophy is an important indicator and often on early clinical sign of significant pathology in the heart. It is an adaptational state to prior hypertension and is a major risk factor associated with heart failure. The cardiac muscle has a large capacity for protein and nucleic acid synthesis since a high degree of turnover of these structural building blocks is necessary for maintenance of the cardiac muscle tissue. The normal process of continuous catabolism of heart proteins and their replacement allows the heart to more rapidly adjust to changes in the demands on the heart. However, when the balance of synthesis and degradation is disturbed, excessive compensation by the

heart in the form of surplus protein and nucleic acid synthesis can lead to cardiac enlargement and compromised contractile function of the heart. Cardiac enlargement may be genetically influenced or may be caused by overworking the heart secondary to disease, pharmacological agents or exercise, and eventually may lead to cardiac failure.

The first signs of cardiac hypertrophy usually are increases in protein and nucleic acid synthesis, as well as other changes in heart metabolism. Because the myocytes of the adult heart rarely undergo mitosis, enlargement of the heart generally is manifested by increases in cardiac muscle cell size rather than cell number. Some of the major symptoms of abnormal cardiac cell growth which can be detected easily in the laboratory include protein content increases, increase in cell size, and accumulation of fibrillar collagen in the extracellular space. Although cardiac muscle cells do not divide, often the connective tissue cells in the heart do increase in number in cardiac enlargement. This increase, and the accumulation of collagen and fibrillar fibronectin in the extracellular matrix lead to myocardial stiffness and ventricular dysfunction. Increase in fibrillar fibronectin is linked to the cell adhesion, migration and growth of cardiac muscle cells which is seen in the typical pattern of cardiac enlargement. It is the interstitial and perivascular fibrosis which accounts for abnormal cardiac stiffness and ultimately ventricular dysfunction.

Primary cultures of cardiomyocytes have been widely used and are recognized as a suitable in vitro model for cardiac hypertrophy at the cellular level.

Hefti et al., J. Mol. Cell Cardiol. 29:2873-2892 (1997). A great many enzymes, growth factors and cytokines have been postulated or shown to influence cardiac hypertrophy, however, the role of 12-lipoxygenase in this phenomenon previously has been undescribed.

Lipoxygenases are enzymes which produce active products, including 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) from arachidonic acid through stereospecific oxygenation. The normal physiological function of these enzymes is not well understood. However, 12-lipoxygenase (12-LO), the enzyme which catalyzes the oxygenation of arachidonic acid to 12(S)-HETE and (S)-12-hydroperoxyeicosatetraenoic acid (12(S)-HPETE), is known to exist in two forms (leukocyte-type and platelet-type) and to play a role in diseases such as atherosclerosis, diabetes and cancer. The mitogenic effects of 12(S)-HETE are similar to those of AII and are abrogated by pertussis toxin, implicating a G-protein mechanism.

12(S)-HETE has direct mitogenic effects in a Chinese hamster ovary (CHO) fibroblast cell line overexpressing the rat vascular type 1a angiotensin II (AT_{1a}) receptor. Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996). These 12(S)-HETE effects mimicked the angiotensin II (AII) mitogenic effects in these cells and led to a sustained increase in DNA synthesis as well as cell number. Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996). Furthermore, the addition of 12(S)-HETE to CHO-AT_{1a} cells led to a significant increase in the activity of the key growth-related kinases, mitogen

activated protein kinases (Wen et al., Am. J. Physiol. 270 (Cell Physiol. 40): C1212-C1220 (1996)), and c-jun amino terminal kinase (Wen et al., Circ. Res. 81:651-655 (1997)). Therefore, angiotensin-II-mediated effects on mitogenesis are likely due to 12-LO products and 12(S)-HETE in particular. The LO pathway also plays a role in the chemotactic effects of platelet-derived growth factor. The products of the 12-LO pathway, are associated with the hypertrophic, hyperplastic, and mitogenic effects induced by AII. Wen et al., 271 Am. J. Physiol. (40 Cell Physiol.) C1212-C1220 (1996); (Natarajan et al., Hypertension 23:I142-I147 (1994)).

Although the precise mechanisms of 12(S)-HETE action are not clear, recent studies have shown that this LO product activates c-jun amino terminal kinase (JNK) (Wen et al., Circ. Res. 81:651-655 (1997)). JNK is a member of the MAP kinase family which is involved in cellular growth, inflammation, and apoptosis (Force et al., Circ. Res. 78:947-953 (1994)) and in cell cycle progression through G_1 (Olson et al., Science 269:1270-1272 (1995)). Evidence shows that JNK can serve as a positive or negative modulator of cell growth in different cells. Olson et al., 269 Science 1270-1272 (1995); Yan et al., 372 Nature 798-800 (1994). Stimulation of the 12-LO pathway in murine macrophages resulted in an increase of monocyte chemotaxis (Scheidegger et al., J. Biol. Chem., 272(34):21609-21615 (1997), presumably through modification of LDL. These activities link AII activation of 12-LO to atherosclerotic disease and cardiac hypertrophy.

Cardiac enlargement and the abnormal cell growth of cells in the heart is a serious health problem. Currently there is no adequate treatment for this condition. Consequently, a new method of treatment of abnormal cardiac cell growth would fill a need in the art.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a method of treating cardiac fibroblast cell growth and hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products. Another embodiment provides a method of preventing cardiac fibroblast cell growth and hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense oligonucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products. Yet another embodiment provides a method of reducing or

eliminating increased protein content in cardiac fibroblasts due to an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said fibroblasts with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense oligonucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the increased protein content resulting from said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a Western blot analysis of cardiac fibroblasts overexpressing 12-LO cDNA.

Figure 2 shows levels of 12(S)-HETE released into the medium bathing 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 3 provides data demonstrating ^{3}H-thymidine incorporation (DNA synthesis) and ^{3}H-leucine labeling (an indicator of protein synthesis) in 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 4 is a set of photomicrographs showing morphological changes in rat cardiac fibroblasts which have been transfected with 12-LO (Figure 4A) compared to mock-transfected rat cardiac fibroblasts (Figure 4B).

Figure 5 shows the forward scatter results of fluorescence-activated cell sorting comparisons of 12-LO-transfected and mock-transfected cells. In each case, 10^{6} cells were sorted.

Figure 6 shows the effect of 12-lipoxygenase overexpression in cardiac fibroblasts on MAP kinases and PAK activities. Arrows on the left side of the Figure indicate the substrates used for measurement of the respective kinase activities indicated at the right.

Figure 7 illustrates the effect of SB202190, a specific p38 MAP kinase inhibitor, on the protein content in 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 8 shows the effect of C_3 transferase pretreatment on ^{3}H-thymidine incorporation by 12-LO-transfected and mock-transfected cells.

Figure 9 shows Northern blot data indicating collagen Iα_1 mRNA levels in 12-LO-transfected and mock-transfected cardiac fibroblasts.

Figure 10 provides data showing the level of soluble fibronectin released into the medium bathing 12-LO-transfected or mock-transfected cardiac fibroblasts and the increase of the fibrillar form of fibronectin resulting from 12-LO overexpression.

Figure 11 shows a significant reduction in cell growth induced by either AII or 12(S)-HETE (0.1μM) by DuP654, a specific 12(S)-HETE receptor blocker.

Figure 12 shows the inhibitory effect on leucine incorporation of baicalein, a specific 12-LO enzyme inhibitor, in cardiac fibroblasts.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Because 12(S)-HETE has several biological effects linked to cellular growth in vascular smooth muscle, CHO AT_{1a} cells and cardiac fibroblasts (Natarajan et

al., Hypertension 23:I142-I147 (1994); Wen et al., Am. J. Physiol. 211:C1212-C1220 (1996)), it is implicated in the etiology of several cardiovascular diseases, including cardiac hypertrophy. Applicants have discovered that 12-LO participates in a previously unknown growth promoting pathway in the heart by discovering that overexpression of 12-LO causes cardiac fibroblast cell growth. The present invention takes advantage of the 12-LO pathway effects which mediate cardiac hypertrophy to provide a new method of treating cardiac fibroblast cell growth and hypertrophy by blocking the expression, activity and/or products of 12-LO.

The harmful effects of 12-LO activation and 12(S)-HETE discussed above are ameliorated by blocking the production of 12(S)-HETE or its binding to specific receptors. For example, 12(S)-HETE receptor blockers include 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol, 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole, pertussis toxin, 12(S)-HETE analogs, antibodies to the 12(S)-HETE receptor and the like. Inhibitors of the 12-LO enzyme include panaxynol, phenidone, pioglitazone, substituted (carboxyalkyl)benzyl ethers, cinnamyl-3,4-dihydroxy-α-cyanocinnamate and the like. Compounds which serve as a structural analog for the enzyme may be useful. Some of these compounds have been described. Gorins et al. (J. Med. Chem. 39:4871-4878 (1996)).

The harmful effects of 12-LO and it products also may be ameliorated by reducing or eliminating 12-LO

expression using antisense or ribozyme methods. Ribozymes which cleave the 12-LO mRNA, preventing its expression, are useful in the invention, and may be constructed according to known methods. Antisense oligonucleotides which bind the 12-LO gene likewise are effective in preventing or reducing 12-LO expression and activity. These oligonucleotides may be constructed using methods known in the art.

To demonstrate the effects of 12-LO on cardiac fibroblasts, 12-LO was overexpressed using the calcium phosphate method in fibroblast cells from neonatal rat heart (kindly provided by Dr. Ping H. Wang, University of California, Irvine). Mouse leukocyte type 12-lipoxygenase cDNA was stably transfected into cardiac fibroblast cells (ML12-LO cells). Cardiac fibroblast cells were maintained in DME medium with 10% FBS containing 20 mM HEPES, pH 7.4, penicillin and streptomycin at 37°C in 5% CO_2 and 95% air. To generate the ML12-LO cells, cardiac fibroblasts were seeded at a density of 1 X 10^6 cells per 100 mm^2 dish. pcDNA1/ML12-LO vector and pPUR vector, a plasmid conferring resistance to puromycin, were cotransfected by the calcium phosphate DNA precipitation method according to manufacturer's instructions (Pharmacia Co.). The 12-LO overexpressing cell line is known as M4.7 cells. Cells transfected with the empty vector pcDNA 1 (Invitrogen) without the 12-LO cDNA insert (P3 cells) were used as a negative, mock-transfected control. Vectors were purified using an Endofree plasmid kit (Qiagen Co.). Forty-eight hours after transfection, the cells were split 1:15. Selection was then initiated with 2 μg/ml of puromycin to select

cells expressing resistance to this marker. Individual resistant clones were isolated 2-3 weeks later and expanded into cell lines. Transfected cells were maintained in medium containing 10% FBS, and 2 μg/ml puromycin.

Immunoblots were used to analyze the expression of the 12-LO protein. A polyclonal antibody against a peptide comprising amino acids 646-662 of porcine leukocyte 12-LO was raised in rabbits. The antibody showed excellent cross-reactivity with murine leukocyte 12-LO. For analysis, cells were lysed in PBS buffer (pH 7.4) containing 1% Triton X-100, 0.1% SDS and a standard protease inhibitor cocktail according to known methods. Lysates were sedimented and the supernatants were collected for assay. Protein concentration was determined by the Bradford method for this assay and all assays disclosed here. The protein (20 μg) was resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gel) and subsequently transferred to a polyvinylidine difluoride membrane. After the membrane was incubated in blocking buffer (Tropix Inc., Bedford) overnight, 12-LO antibody was added at a 1:1000 dilution. After incubation, an alkaline phosphatase coupled goat anti-rabbit secondary antibody was added at a 1:10,000 dilution. The protein bands were visualized using a chemiluminescence substrate and the Western Light Chemiluminescent detection system (Tropix Inc., Bedford). Figure 1 shows the Western blot analysis of these transfected cells. The results indicate several positive clones. It is clear that the M4.7 and M19 clones express much higher steady-state levels of the 12-LO protein compared to

mock-transfected clone P3 in which 12-LO protein was only slightly detectable. Clone M4.7 showed the greatest overexpression of 12-LO compared to the mock-transfected P3 cell line. (See Figure 1).

To evaluate cellular hypertrophy, these two cell lines (M4.7 and P3) were labeled with either ^{3}H-thymidine to evaluate DNA synthesis or with ^{3}H-leucine as an indicator of protein synthesis. For determination of ^{3}H-thymidine, ^{3}H-leucine or ^{3}H-uridine incorporation and protein content, cardiac fibroblast cells were grown in 6-well culture plates for 3 days. After twenty to twenty-four hours depletion with DME medium containing 20 mM HEPES, pH 7.4, 0.2% bovine serum albumin (BSA) and 0.4% fetal bovine serum (FBS), cells were continuously cultured in depletion medium containing 1 μCi/ml ^{3}H-thymidine, ^{3}H-leucine or ^{3}H-uridine. Approximately 24 hours later, the ^{3}H-isotope incorporation and protein content were measured. The medium was aspirated, cells were washed twice with 1 ml cold PBS solution and once with 1 ml 10% trichloroacetic acid (TCA). The cells were then incubated in fresh 1 ml 10% TCA at 4°C for 30 minutes. The TCA-insoluble material was washed twice with 95% ethanol. Fixed cellular material was solubilized in 0.1 N NaOH at 24°C for 2 hours. The sample was divided into 6 wells (3 wells for incorporated and protein content measurements; 3 wells for cell counting). The ^{3}H-isotope incorporation was determined by liquid scintillation spectrometry. Cells were counted with a Coulter Counter. The data was normalized as cpm/10^6 cells or μg protein/10^6 cells and expressed as fold increase over mock-transfected controls.

The data presented in Figure 2 show that 12-LO transfected cardiac fibroblasts release into the medium about 4 times the level of 12(S)-HETE than mock-transfected cells. 12(S)-HETE concentrations were measured by a specific radioimmunoassay with a sensitivity of 10 pg/ml and intraassay variation of 8%.

The results of ^{3}H-thymidine and ^{3}H-leucine labelling are shown in Figure 3. ^{3}H-Leucine incorporation is shown in the left panel. The overexpression of 12-LO resulted in increases in leucine incorporation about 2 fold (2.1 ± 0.1 fold; n=4; $p < 0.01$) over that in mock-transfected cells. The data in the right panel show that ^{3}H-thymidine incorporation increased more than 3 fold (3.4 ± 0.3 fold; n=4; $p < 0.01$) in cells overexpressing 12-LO compared to mock-transfected cells. Incorporation of ^{3}H-uridine in these 12-LO-transfected cells was over 5 fold greater than that seen in control cells (5.6 ± 0.7 fold; n=3; $p < 0.01$; data not shown). Protein content measurements indicated that cardiac fibroblasts overexpressing 12-LO contained 2.2 ± 0.3 fold more protein than controls when expressed as $\mu g/10^6$ cells (n=5; $p < 0.01$; data not shown). 12-LO expression did not lead to a true increase in cell number, however, these data clearly show that 12-LO products can result in dramatic increases in cell anabolism.

To further analyze hypertrophic effects of 12-LO overexpression, cell size was examined using hematoxylin eosin staining on cells in chamber slides. Cell size, nucleus size and nucleolus count were quantitated in mock- (Figure 4B) and 12-LO-transfected (Figure 4A) cells. Most of the mock-transfected cells

have one nucleolus. In contrast, 12-LO-transfected cells at the same magnification are bigger, the nuclei are larger and interestingly, they have a higher average number of nucleoli than control cells. See Figure 4. To quantiate this effect, twenty nuclei in Figure 4 were arbitrarily chosen and the long axis and numbers of nucleoli were measured. The data showed that the mean long axis of nuclei in 12-LO transfected cells was 6.4 ± 0.21 mm or nm compared to 4.15 ± 0.26 mm or nm in control cells. These cells were cultured in chamber slides and incubated at 37°C for 24 hours. After washing with PBS, the cells were fixed in 100% acetone and then stained with HE staining (hematoxylin for 5 minutes and eosin for 1 minute). The results indicate that the mean long axis of nuclei in 12-LO transfected cells was about 1.54 fold greater than that in control cells ($p < 0.001$). The analysis also demonstrated that the mean number of nucleoli was 2.95 in 12-LO transfected cells versus 1.5 in mock-transfected cells ($p < 0.001$). These results suggest that 12-LO overexpression leads to significant morphological changes consistent with cellular hypertrophy in rat cardiac fibroblasts.

The cell size increase in 12-LO transfected cells was confirmed using FACS analysis. The left panel of Figure 5 illustrates the forward scatter histogram of control and 12-LO transfected cells. The right panel of Figure 5 shows both 12-LO overexpressing and mock-transfected cells redrawn using a computer program. The average size of the 12-LO overexpressing cells was shifted to the right compared to that of control cells.

The effect of 12-LO overexpression on MAP kinases and PAK was evaluated since these signaling enzymes have been linked to cell growth, cell apoptosis and focal adhesion activity. ERK1/2, JNK-1 and p38 activity was measured with an immune complex kinase assay according to methods known in the art. Growth of the cells was arrested by incubation in depletion DME medium containing 0.2% BSA, 0.4% FCS, 20 mM HEPES, pH 7.4, for 24 hours. After washing twice with cold PBS, the cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 50 mM NaF, 10 mM Na pyrophosphate, 2.5% glycerol, 1% NP-40 and 1 mM Na_3VO_4 and the protease inhibitors PMSF, leupeptin and aprotinin. The lysate was sedimented at 14,000 xg at 4°C for 10 minutes. The lysate protein (50 μg) was immunoprecipitated with JNK, p38 or ERK antibodies in lysis buffer and the mixture incubated under rotation at 4°C overnight, followed by addition of the solution to 60 μl protein A Sepharose beads. After 1 hour incubation with protein A Sepharose at 4°C, the beads were washed 4 times with buffer and pelleted. The pelleted beads were resuspended in 60 μl kinase buffer containing substrates as follows: 2 μg GST-c-Jun(aa 1-79) for the JNK assay, 2 μg ATF-2 for the p38 assay or 2 μg MBP for the ERK assay and 20 μM ATP containing 5 μCi [γ-^{32}P]ATP. After 30 minutes at 30°C, the reaction was stopped and samples were resolved on 12% SDS-polyacrylamide gels followed by autoradiography.

The protein kinase activity studies showed that overexpression of 12-LO enhanced extracellular signal-regulated kinase (ERK) activity, cJun NH_3-terminal kinase (JNK) activity, p38 MAP kinase (p38)

activity and p21-activated kinase (PAK) activity. See Figure 6. These data clearly demonstrate that overexpression of 12-LO causes cardiac fibroblast cell growth, showing that 12-LO participates in a previously unknown growth promoting pathway to the heart. The data in Figure 6 clearly show that the overexpression of 12-LO results in activation of JNK, ERK, p38 and PAK. Overexpression of 12-LO stimulated ERK 4.1 ± 0.5 fold ($n=3$; $p < 0.01$), p38 2.2 ± 0.3 fold ($n=3$; $p < 0.02$) and JNK 2.9 ± 0.5 fold ($n=3$, $p < 0.02$), respectively.

Figure 7 presents data showing the effect of SB202190, a specific p38 MAP kinase inhibitor, on the protein content of cardiac fibroblast cells. Comparing the data for the 12-LO transfected cells and mock-transfected controls shows that the overexpression of 12-LO results in increases in protein content of about 2 fold. The p38 inhibitor had no significant effect on protein content in mock-transfected cells, implying that p38 MAP kinase activation may be important for 12-LO-induced protein content increases, but completely blocked the protein content increase induced by 12-LO overexpression. In contrast, the compound PD58059, an inhibitor of MEK, had no effect on leucine incorporation either in 12-LO-transfected or mock-transfected cells (data not shown). This clearly demonstrates the effect that abnormally increased 12-LO expression has on cardiac fibroblasts, and the relationship of 12-LO to p38 MAP kinase, a known enzyme in the signal pathway which causes cardiac enlargement.

Previous evidence has shown that arachidonic acid and its metabolites can activate small molecular weight

GTP-binding proteins. To elucidate the effect of the small molecular weight GTP-binding protein, Rho, on ^{3}H-thymidine incorporation, M4.7 and P3 cardiac fibroblasts were pretreated with the specific Rho GTPase inhibitor, ADP-ribosyltransferase C_3 (C_3 transferase; 10 μg/ml) for 24 hours. C_3 transferase treatment reduced DNA synthesis by 34% in the 12-LO overexpressing cardiac fibroblast cells but not in mock transfected cardiac fibroblast cells (see Figure 8). These results suggest that 12-LO-induced increases in cardiac fibroblast growth is mediated at least in part by activation of small molecular weight GTP-binding proteins such as Rho.

A collagen Iα_1 cDNA probe was used to detect collagen mRNA content in these cells as an indicator of another symptom in cardiac hypertrophy. Figure 9 illustrates the results. As shown in the top panel, the Northern Blot detected two collagen bands at 4.6 kb and 5.6 kb. This is consistent with the published data for this probe. The lower two panels show the densities of these two bands indicating that the overexpression of 12-LO increased mRNA content of these two bands about 4 fold over that in mock-transfected cells.

To evaluate the effect of 12-LO expression on matrix production, soluble fibronectin released into the medium of cardiac fibroblasts transfected or mock-transfected with 12-LO was measured with a specific ELISA assay as yet another test of the relationship between 12-LO and cardiac hypertrophy. Because the presence of fibrillar fibronectin is more closely related to cardiac hypertrophy, measurements

were taken not only for fibronectin released into medium (soluble fibronectin), but also 1% deoxycholate insoluble fibronectin (fibrillar fibronectin).

Cells at 80% to 90% confluence were depleted with medium containing 0.2% BSA and 0.4% FCS for 24 hours. The supernatants were assayed for released fibronectin. Washed cell layers were extracted with 1% deoxycholate. Deoxycholate extractions were performed in 0.02 M Tris buffer, pH 8.3, containing 2 mM phenylmethylsulfonilfluoride (PMSF), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylmaleimide and 2 mM iodoacetic acid. For sequential extraction, cell layers were scraped into 1% deoxycholate and sedimented at 17,000 rpm for 20 minutes. Deoxycholate-insoluble material was solubilized at 70°C in 4% SDS. The fibronectin in supernatants was classified as soluble fibronectin, while the 1% deoxycholate-insoluble material was classified as fibrillar. Fibronectin in all samples was determined by a double-antibody sandwich enzyme-linked immunosorbent assay using the methods provided by the manufacturer (DACO Corp., Carpinteria, CA). A polyclonal rabbit anti-human fibronectin (1:1000) was used as the coating antibody, and the detection antibody was a peroxidase-conjugated rabbit anti-human fibronectin (1:2000).

The results show that overexpression of 12-LO increases soluble fibronectin release into the medium about 3.7 fold over that in mock-transfected cells ($p < 0.01$) and increases fibrillar fibronectin 3.4 times compared to mock-transfected cells ($p < 0.01$). See Figure 10. This suggests that more surface-bound

fibronectin assembled into large fibrils in cells overexpressing 12-LO.

The results of these experiments on cell protein content, cell size, collagen production and fibrillar fibronectin clearly indicate that overexpression of 12-LO causes both cardiac fibroblast cell hypertrophy and matrix production, and demonstrate that 12-LO participates in a heretofore unrecognized growth-promoting pathway in the heart. The present invention therefore provides a method of treating or preventing abnormal cardiac cell growth by inhibiting the effects of 12-LO and its products. This may be accomplished by antagonizing or blocking the receptors for the 12-LO product, 12(S)-HETE, with a 12(S)-HETE receptor blocker such as 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 12(S)-HETE receptors, antibodies to the 12(S)-HETE receptor and the like. Reducing or eliminating the production of 12(S)-HETE through a 12-LO enzyme inhibitor, for example, panaxynol, a polyacetylene compound isolated from ginseng (Powell et al., Science 245:186-188 (1989)), phenidone, 5,6,7-trihydroxyflavone (baicalein), pioglitazone, substituted (carboxyalkyl)benzyl ethers such as those disclosed in Gorins et al., J. Med. Chem. 39:4871-4878 (1996), cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) and the like also forms part of the inv ntion.

Expression of 12-LO enzyme may be prevented using antisense or ribozyme methods according to the invention. Antisense oligonucleotides which bind 12-LO genetic material are useful to prevent or reduce 12-LO activity in cardiac fibroblasts. This invention therefore provides a method of controlling cardiac cell growth and hypertrophy employing antisense. Oligonucleotides may be constructed using any suitable method known in the art. Ribozymes designed to specifically cleave 12-LO mRNA are provided by the present invention. Methods known in the art may be used to construct ribozymes which bind and cleave 12-LO mRNA. He cardiac cell growth and hypertrophic effects of 12-LO are thereby reduced or eliminated.

The determination of appropriate, well-tolerated dosage forms for administration to humans for use in the present invention is within the ordinary skill in the art. Such dosage forms include tablets, capsules, syrups, suspensions, drops, injectable solutions, lozenges, implants, transdermal patches, and other dosage forms well known in the art for enteral or parenteral administration. Based on in vitro experiments on the effect of 12(S)-HETE receptor blocking drugs on 12(S)-HETE binding, a dose of between about 0.5 and about 30 mg/kg/day would be effective in blocking 12(S)-HETE receptors in humans in vivo, and preferably from about 1 to about 10 mg/kg/day. Similar dosages may be given when 12-LO inhibitors are used. Methods of administering ribozymes or antisense oligonucleotides may be designed according to any suitable known method.

EXAMPLES

Example 1

Reduction of cell growth induced by AII or 12(S)-HETE.

DuP654, a selective 12(S)-HETE receptor antagonist, significantly reduced cell growth induced by either AII or 12(S)-HETE at a concentration of 0.1 mM in CHO-AT_{1a} cells *in vitro*. Complete inhibition of 12(S)-HETE induced mitogenic effects was seen. See Figure 11.

Example 2

Inhibition of serum-induced leucine incorporation by cardiac fibroblasts.

Baicalein, a specific inhibitor of 12-lipoxygenase, inhibited leucine incorporation in cardiac fibroblasts. The inhibition was both highly significant and dose-dependant ($P<0.005$ at 1μM; $P<0.001$ at 10 μM). See Figure 12. These data clearly demonstrate that blockade of the 12-LO pathway meaningfully reduces the indicia of cell growth in mammalian cardiac fibroblasts.

CLAIMS

1. A method of treating cardiac fibroblast cell growth and hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

2. The method according to claim 1, wherein the compound is a 12-lipoxygenase inhibitor.

3. The method according to claim 2, wherein the 12-lipoxygenase inhibitor is selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl-3,4-dihydroxy-α-cyanocinnamate.

4. The method according to claim 1, wherein the compound is a 12(S)-HETE receptor blocker.

5. The method according to claim 4, wherein the 12(S)-HETE receptor blocker is selected from the group consisting of 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having

affinity for the binding site on the 12(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

6. The method according to claim 1, wherein the compound is a 12-lipoxygenase antisense nucleotide.

7. The method according to claim 1, wherein the compound is a 12-lipoxygenase ribozyme.

8. A method of controlling cardiac fibroblast cell growth and preventing hypertrophy in a cell having an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said cell with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme, and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the cardiac fibroblast cell growth and hypertrophic effects of said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

9. The method according to claim 8, wherein the compound is a 12-lipoxygenase inhibitor.

10. The method according to claim 9, wherein the 12-lipoxygenase inhibitor is selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl-3,4-dihydroxy-α-cyanocinnamate.

11. The method according to claim 8, wherein the compound is a 12(S)-HETE receptor blocker.

12. The method according to claim 11, wherein the 12(S)-HETE receptor blocker is selected from the group consisting of 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 12(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

13. The method according to claim 8, wherein the compound is a 12-lipoxygenase antisense nucleotide.

14. The method according to claim 8, wherein the compound is a 12-lipoxygenase ribozyme.

15. A method of reducing or eliminating increased protein content in cardiac fibroblasts due to an excess of 12-lipoxygenase activity or 12-lipoxygenase products, comprising contacting said fibroblasts with a compound selected from the group consisting of a 12(S)-HETE receptor blocker, a 12-lipoxygenase antisense nucleotide, a 12-lipoxygenase ribozyme and a 12-lipoxygenase inhibitor in an amount effective to reduce or eliminate the increased protein content resulting from said excess of 12-lipoxygenase activity or 12-lipoxygenase products.

16. The method according to claim 15, wherein the compound is a 12-lipoxygenase inhibitor.

17. The method according to claim 16, wherein the 12-lipoxygenase inhibitor is selected from the group consisting of pioglitazone, panaxynol, phenidone, baicalein, and cinnamyl-3,4-dihydroxy-α-cyanocinnamate.

18. The method according to claim 15, wherein the compound is a 12(S)-HETE receptor blocker.

19. The method according to claim 18, wherein the 12(S)-HETE receptor blocker is selected from the group consisting of 13(S)-hydroxyoctadecadienoic acid, 2-phenylmethyl-1-naphthol (DuP654), 2-N-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole (Losartan), pertussis toxin, 12(S)-HETE analogs, peptides and peptide analogs having affinity for the binding site on the 12(S)-HETE receptors and antibodies to the 12(S)-HETE receptor.

FIG. 1

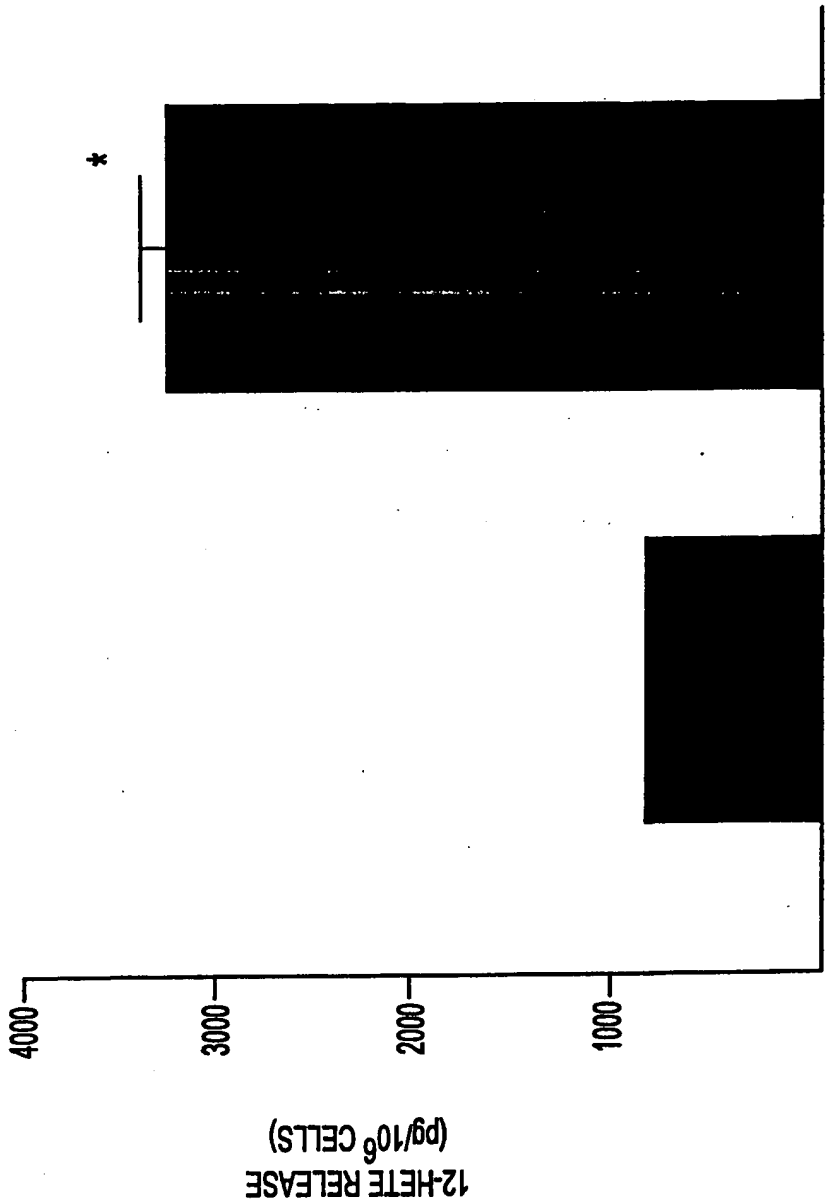

FIG. 2

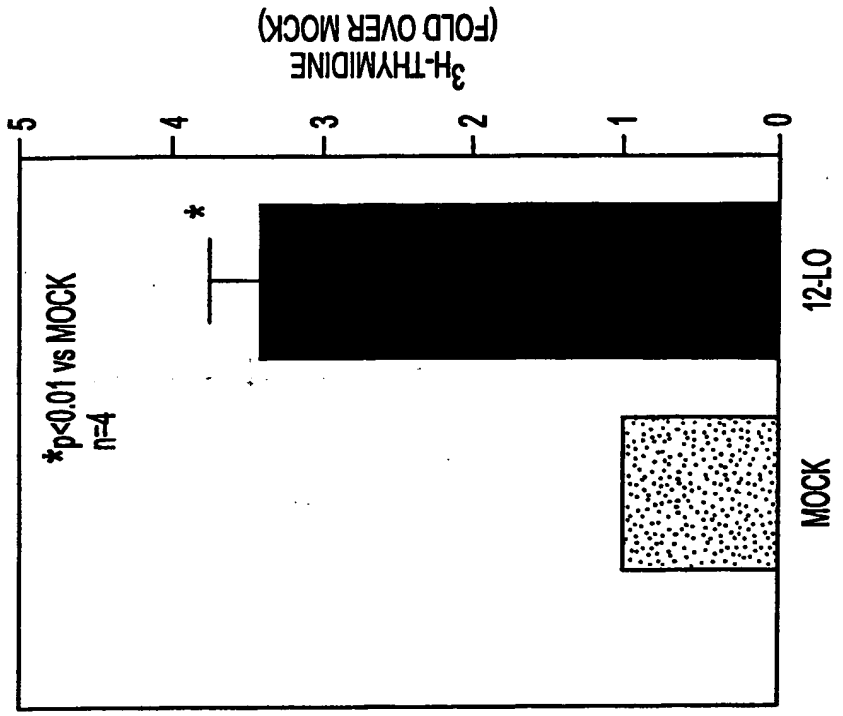

FIG. 3B

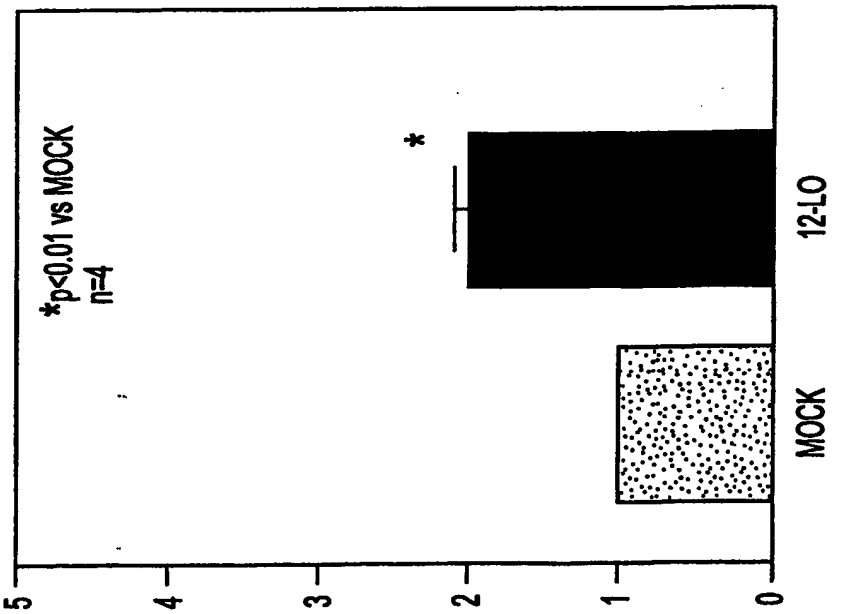

FIG. 3A

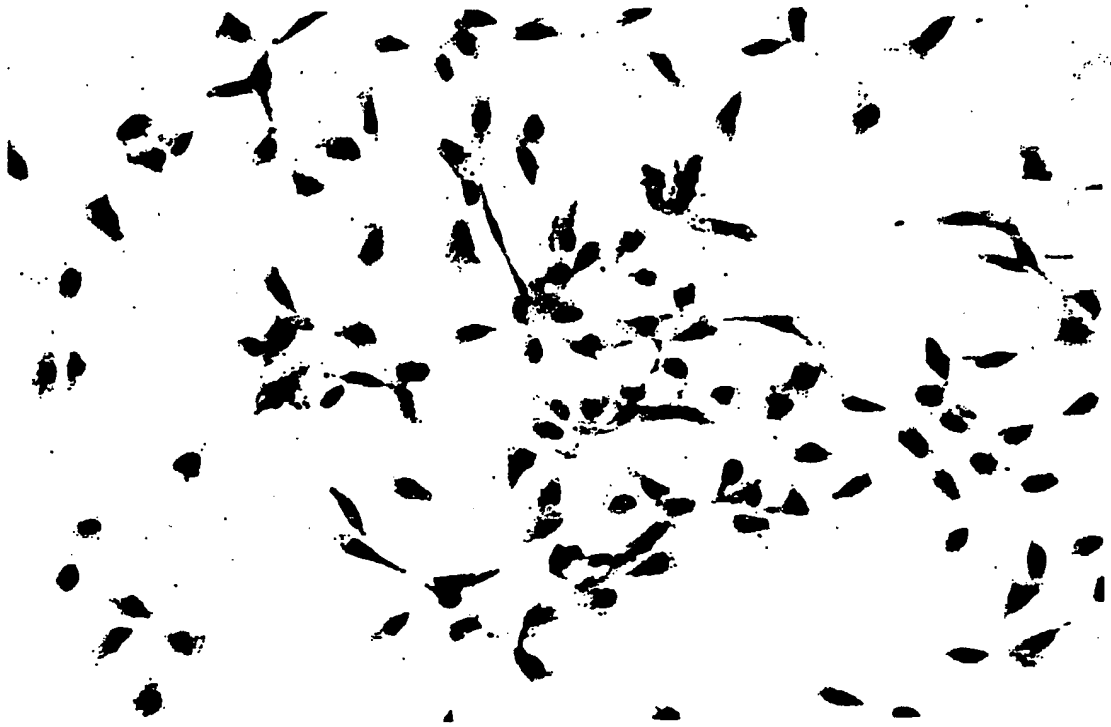

FIG. 4A

FIG. 4B

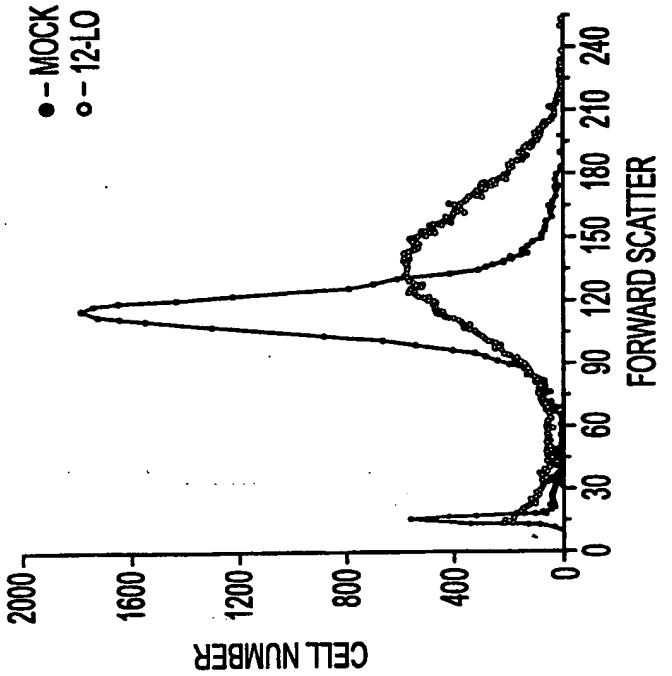

FIG. 5C

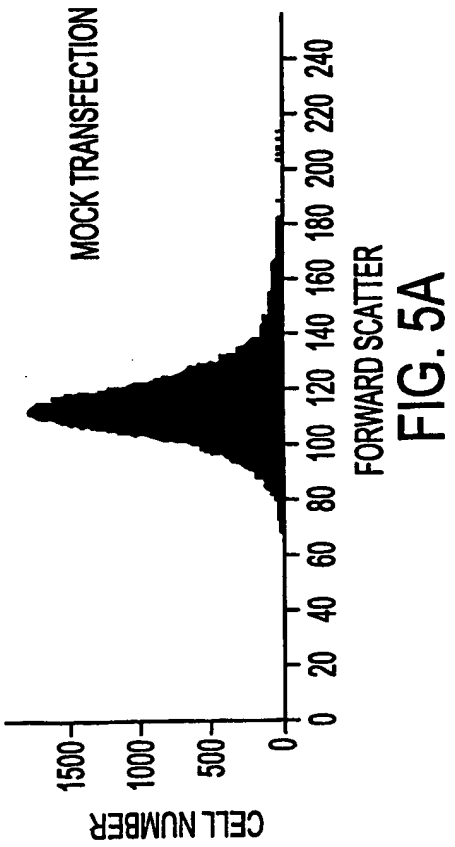

FIG. 5A

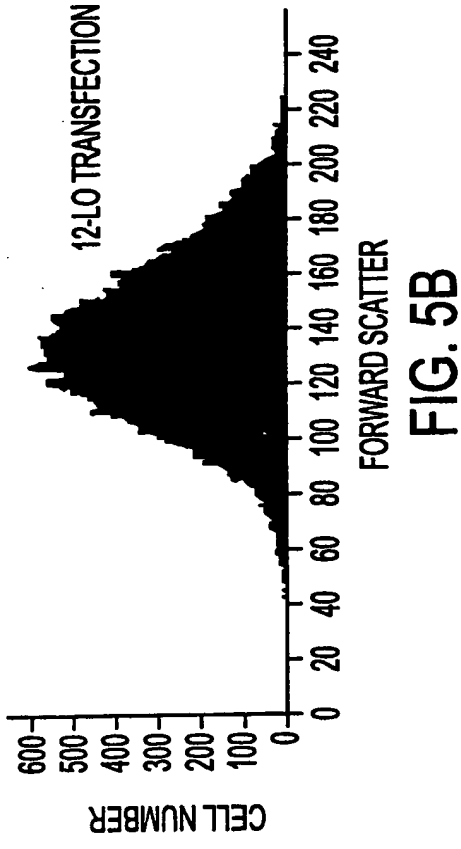

FIG. 5B

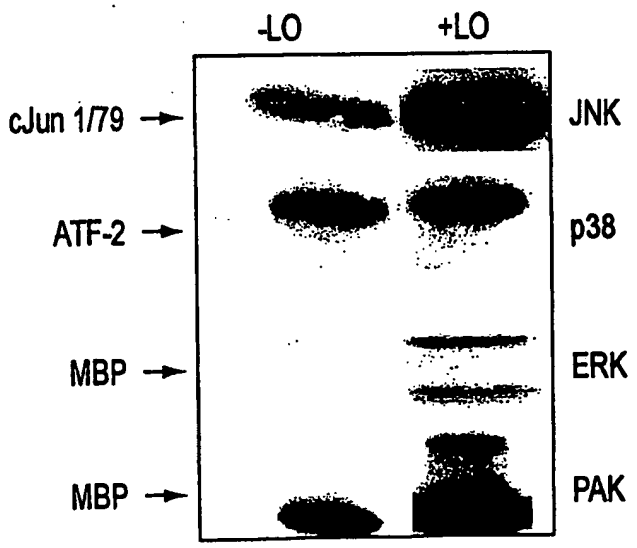

FIG. 6

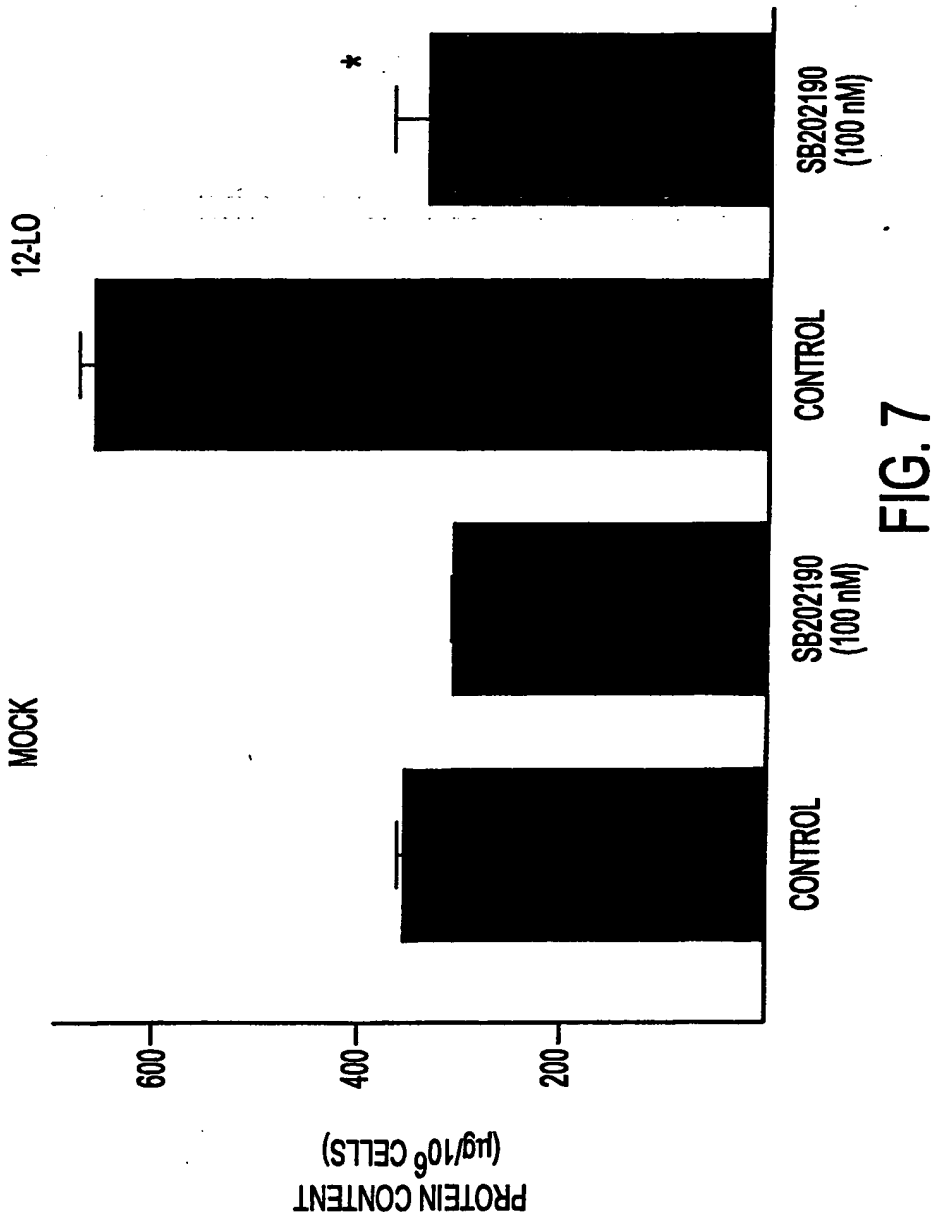

FIG. 7

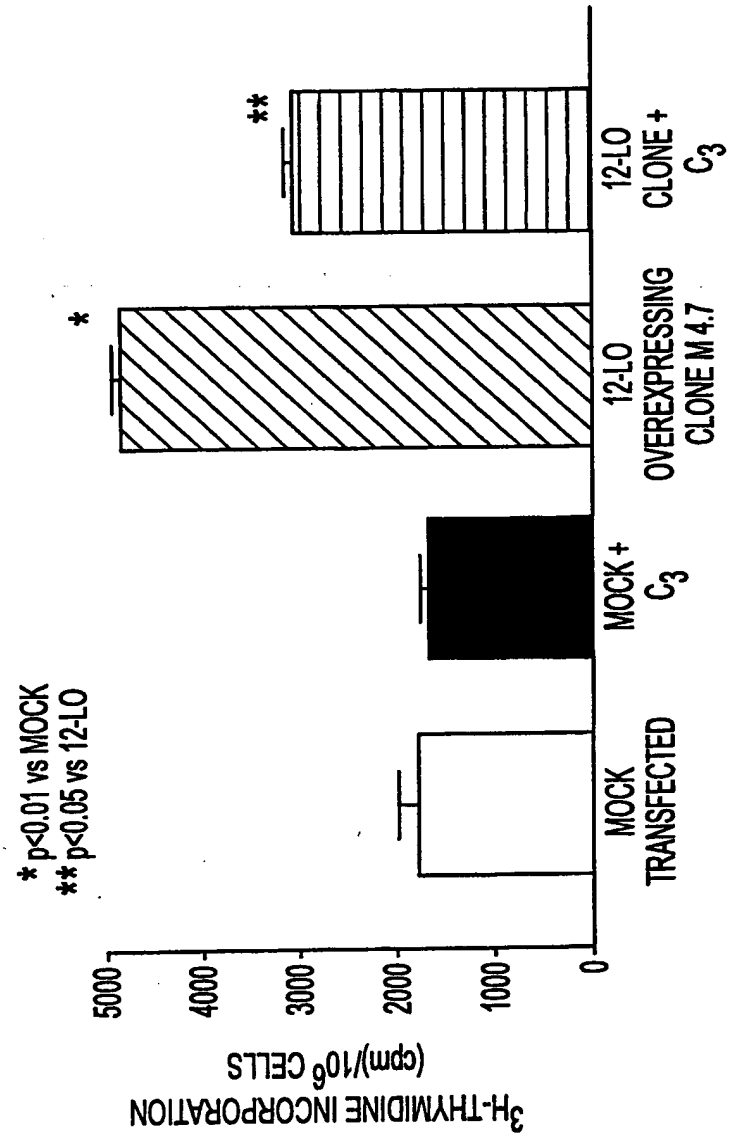

FIG. 8

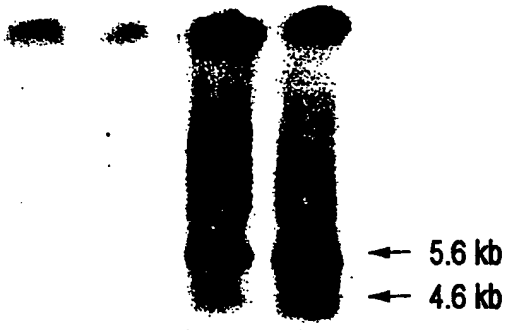

FIG. 9A

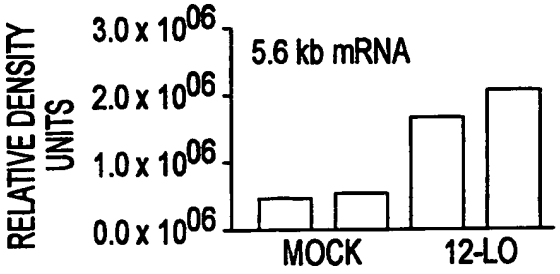

FIG. 9B

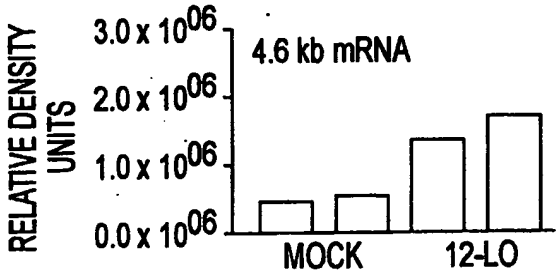

FIG. 9C

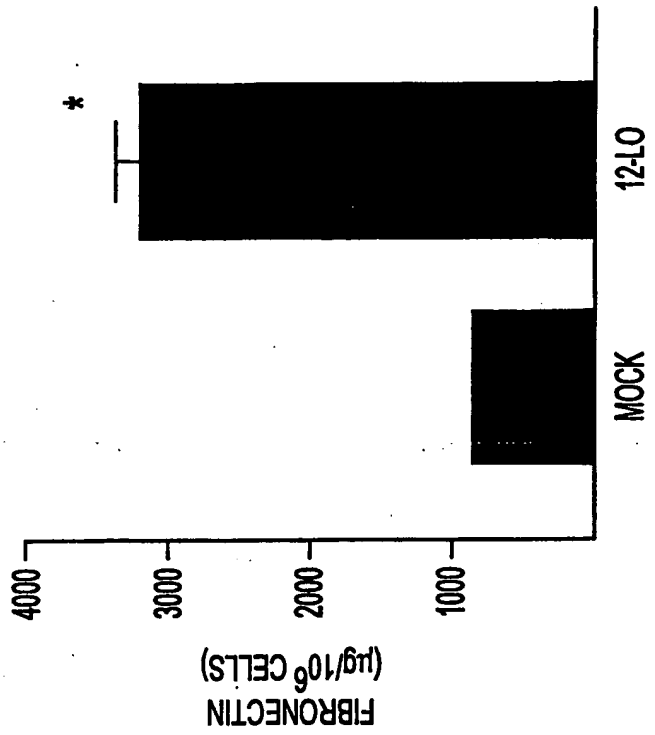

FIG. 10B

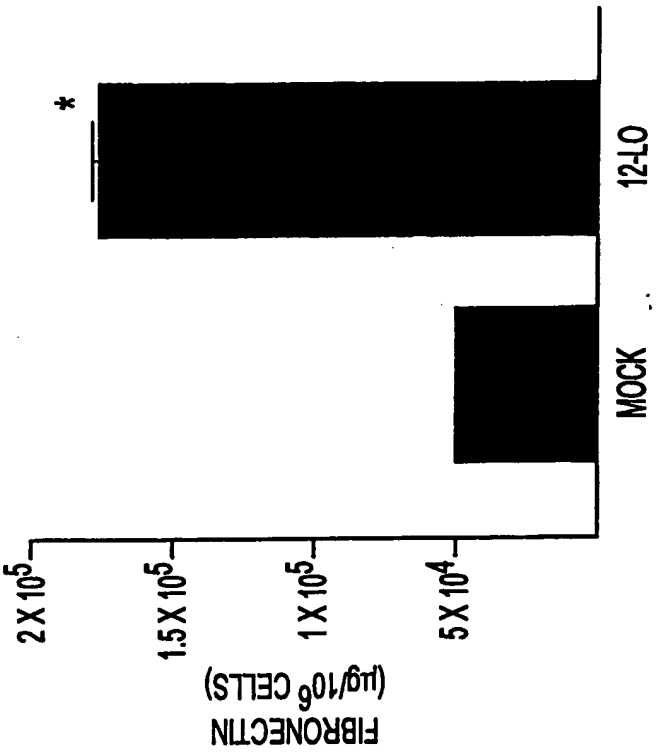

FIG. 10A

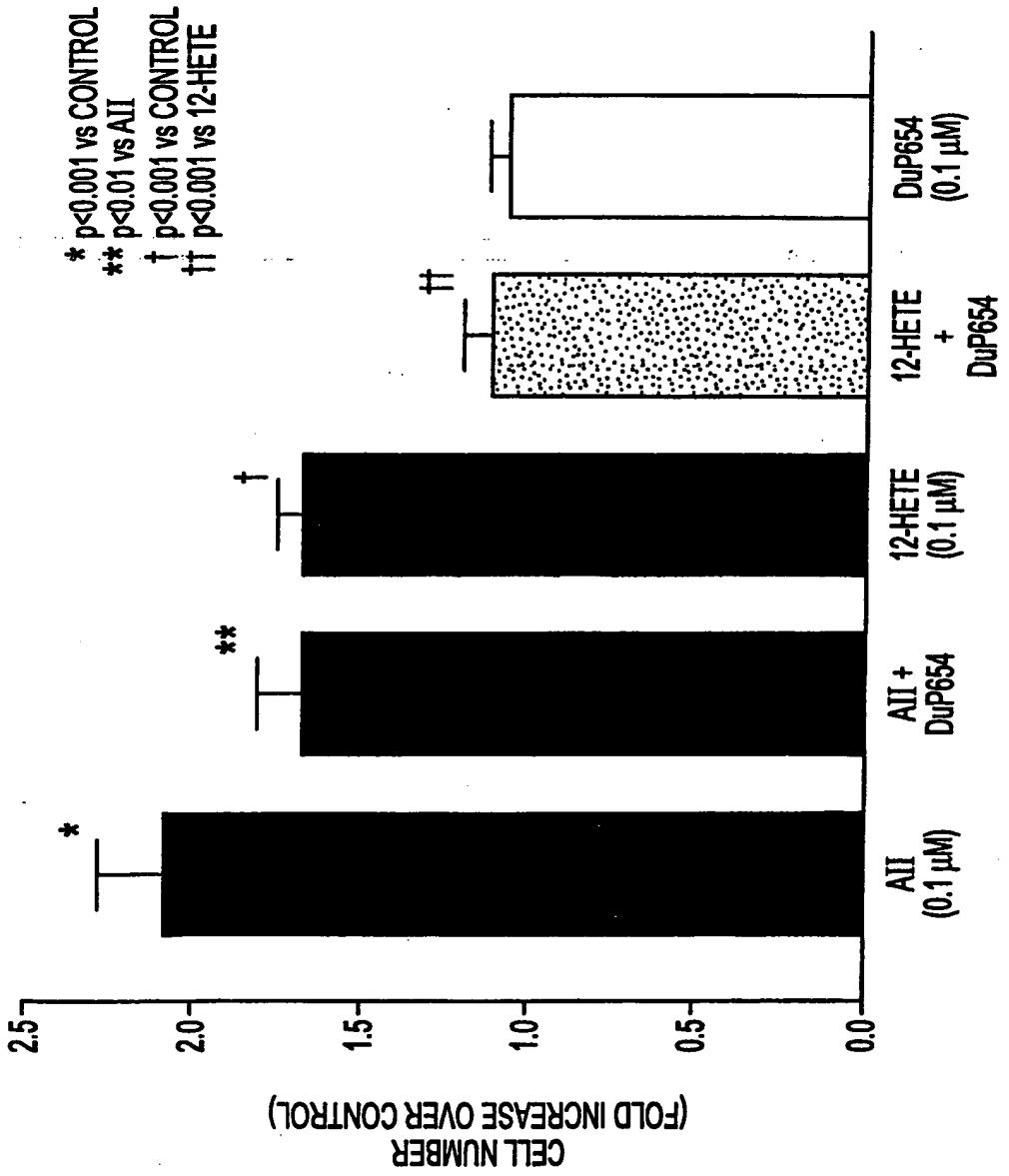

FIG. 11

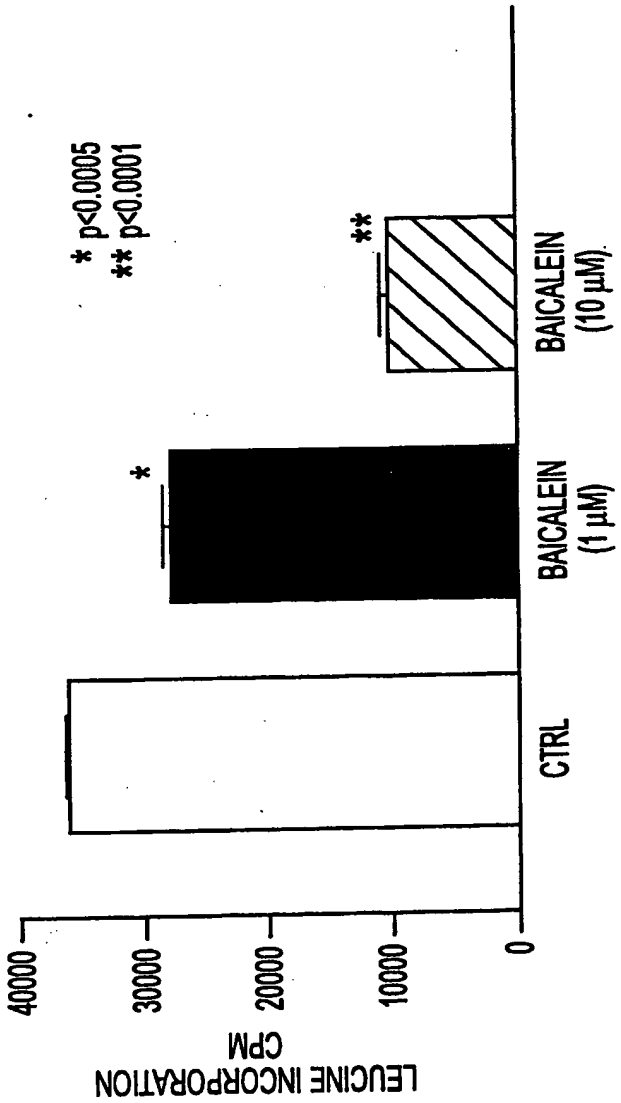

FIG. 12

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/11115

A. CLASSIFICATION F SUBJECT MATTER
IPC 6 A61K31/44 A61K31/415 A61K31/35 A61K31/275 A61K31/20
A61K31/05 A61K31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BROTEN ET AL.: "Losartan and enalapril prevent cardiac hypertrophy and intramural coronary arterial hypertrophy and fibrosis in a low renin model of hypertension." FASEB JOURNAL, vol. 8, no. 4-5, 1994, page A310 XP002117416 abstract --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 November 1999	- 8. 02. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	A. Jakobs

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 99/11115

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DOMINICZAK A. F.; DEVLIN A. M.; LEE W. K.; ANDERSON N. H.; BOHR D. F.; REID J. L.: "Vascular smooth muscle polyploidy and cardiac hypertrophy in genetic hypertension." HYPERTENSION, vol. 27, no. 3 pt 2, 1996, pages 752-759, XP002117417 abstract; figures 1,3,4 page 756, column 2, paragraph 1 ---	1
X	GUO, ZHAO-GUI; LING, QI; SU, ZHI: "Mechanism of losartan in the regression of cardiac hypertrophy." FASEB JOURNAL, vol. 12, no. 5, 1998, page a709 XP002117418 abstract ---	1
X	NATARAJAN, RAMA; GONZALES, NOE; LANTING, LINDA; NADLER, JERRY: "Role of the lipoxygenase pathway in angiotensin II-induced vascular smooth muscle cell hypertrophy." HYPERTENSION, vol. 23, no. 1, 1994, pages I142-I147, XP002117419 abstract; figures 2-4 page I145, column 1, paragraph 3 -page I146, column 1, paragraph 2 ---	1,4,5,8, 11,15, 18,19
X	DETHLEFSEN, SANDRA M. ; SHEPRO, DAVID; D'AMORE, PATRICIA A.: "Arachidonic acid metabolites in bFGF-, PDGF-, and serum-stimulated vascular cell growth." EXPERIMENTAL CELL RESEARCH, vol. 212, no. 2, 1994, pages 262-273, XP002117420 abstract page 262, column 2, paragraph 3 -page 263, column 1, paragraph 1; figures 1-12 see results ---	1,4,5,8, 11,15, 18,19
X	WO 96 40256 A (SEARLE & CO ;MCMAHON ELLEN G (US); OLINS GILLIAN M (US); SCHUH JOS) 19 December 1996 (1996-12-19) page 8, line 3 -page 11, line 27 page 130, compound 325 --- -/--	1

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 99/11115

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Structure-activity relationship for potentiation of EGF-dependent mitogenesis by oxygenated metabolites of linoleic acid" ARCH. BIOCHEM. BIOPHYS. (1994), 311(2), 286-92, XP002117421 abstract page 286, column 1, paragraph 1 -page 287, column 1, paragraph 2 page 288, column 1, paragraph 1 -page 289, column 2, paragraph 1; figure 2; table 1 ---	1,4,5,8, 11,15, 18,19
A	BAILEY ET AL.: "15-lipoxygenase induction as an index of oxidative stress and atherogenesis" BIOCHEM. SOC. TRANS., vol. 21, no. 4, 1993, page 406S XP002117422 the whole document ---	1,4,5,8, 11,15, 18,19
X	EP 0 339 671 A (SUNTORY LTD) 2 November 1989 (1989-11-02) page 2, line 1-50 ---	1,4
X,P	YESHAO, WEN ; GU, JIALI ; WANG, PING H.; NADLER, JERRY L.: "Overexpression of 12 lipoxygenase causes cardiac fibroblast cell growth." HYPERTENSION, vol. 32, no. 3, 1998, page 630 XP002117423 abstract ---	1
P,X	WO 99 18956 A (HOPE CITY) 22 April 1999 (1999-04-22) page 19, line 7-17; example 2 ---	1,2,4
X	US 5 102 912 A (STREBER AUGUST S) 7 April 1992 (1992-04-07) column 2, line 12 -column 4, line 5; claims 1,6,8 -----	1,4,5,8, 11,12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 11115

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-19 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,4,5,8,11,15,18,19 (all partially)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of 13-(S)-hydroxyoctadecadienoic acid and other 12-(S)-HETE analogs for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

2. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of 2-phenylmethyl-1-naphthol for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

3. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of losartan for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

4. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of pertussis toxin for treating and controlling cardiac fibroblast cell growth and hypertrophy in a cell.

5. Claims: 1,4,5,8,11,15,18,19 (all partially)

   Use of peptides and peptide analogs having affinity for the binding site one the 12 (S)-HETE receptor and antibodies to the 12(S)-HETE receptor for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

6. Claims: 1 (partially), 6, 8 (part.), 13, 15 (part.)

   Use of a 12-lipoxygenase antisense nucleotides for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

FURTHER INFORMATION CONTINUED FR M PCT/ISA/ 210

7. Claims: 1 (partially), 7, 8 (part.), 14

Use of a 12-lipoxygenase ribozyme for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

8. Claims: 1-3,8-10,15-17 (all partially)

Use of pioglitazone for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

9. Claims: 1-3,8-10,15-17 (all partially)

Use of panaxynol for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

10. Claims: 1-3,8-10,15-17 (all partially)

Use of phenidone for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

11. Claims: 1-3,8-10,15-17 (all partially)

Use of baicalein for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

12. Claims: 1-3,8-10,15-17 (all partially)

Use of cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate for treating and controlling cardiac fibroblast cell growth and hypertrophy of a cell.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/11115

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640256 A	19-12-1996	AU 5982296 A	30-12-1996
EP 0339671 A	02-11-1989	JP 2235852 A	18-09-1990
		JP 2647185 B	27-08-1997
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		AT 109770 T	15-08-1994
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